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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 muco-inert 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

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formulations for vaginal drug delivery via MPP may significantly improve prevention and treatment of reproductive tract diseases and disorders.

Keywords

Mucus-penetrating particles; hypotonic delivery; microbicides

Introduction

Vaginal products have traditionally been made with *hypertonic* 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 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.

Materials and Methods

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 μ L 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.

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.

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 isototically and hypototically 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 hr 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 a 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 μ l 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 μ m 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 μ l 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 μ l 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.

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 μ l 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.

Red fluorescent MPP were suspended in either PBS (isotonic) or ultrapure water (hypotonic) at 0.2% (w/v). Mice were either anesthetized with an intraperitoneal injection of Avertin (“non-ambulatory”) or by inhalation of isoflurane (“ambulatory”) as described previously

for drug distribution experiments. Five μl of either isotonic or hypotonic MPP solution was administered intravaginally. After 1 h for non-ambulatory mice, or 10 min for ambulatory mice, whole cervicovaginal tracts were excised and placed in a standard tissue culture dish. Fluorescence images were taken and quantified as described for drug retention. As a reference point for MPP retention, 5 μl of MPP solution was carefully pipetted into the vagina after the whole cervicovaginal tract was already removed and placed in a tissue culture dish. This approach was used for MPP (but not Doxorubicin), because the nanoparticles do not penetrate the tissue; therefore there is no potential effect from differential tissue penetration on the fluorescent signal. The retention was then calculated as a percentage of the average reference tissue signal.

HSV-2 infection mouse model

Mice received 20 μl of 10 mg/ml acyclovir monophosphate dissolved in either PBS (isotonic) or ultrapure water (hypotonic) immediately before use. This drug was shown to give partial protection in a mouse model of vaginal HSV-2 infection [16]. Mice were dosed either 1 min or 60 min prior to viral inoculum. Mice were then challenged with 10 μl of inoculum containing HSV-2 strain G (ATCC #VR-724, 2.8×10^7 TCID₅₀ per ml). For protection studies, HSV-2 was diluted 10-fold with Bartel's medium to deliver 10 ID₅₀, a dose that typically infects 85-90% of control mice. For osmotic-induced susceptibility tests, virus was diluted 10-fold with Bartel's medium and further diluted 10-fold in either Bartel's medium (isotonic) or deionized water (hypotonic) to the ID₅₀, a dose that infects half of the mice. Mice were assessed for infection three days later after inoculation by culturing a PBS vaginal lavage on human foreskin fibroblasts (Diagnostic Hybrids, MRHF Lot #440318W), as described previously [20]. In this model, input (challenge) virus is no longer detectable in lavage fluid if it is collected more than 12 h after the challenge.

Statistics

The Wilcoxon rank-sum test was used to compare data sets. This test is non-parametric, and more appropriate in situations where a Gaussian distribution cannot be assumed. For HSV-2 infection studies, statistical significance was determined using Fisher's exact test, two-tailed distribution.

Results

Effect of tonicity on vaginal drug distribution

To investigate the effect of solution tonicity on vaginal drug distribution, we used Doxorubicin (Dox) since it is fluorescent and is used for treating female reproductive tract cancers. In groups of mice that were non-ambulatory (supine for 1 h), only $15 \pm 2\%$ of the vaginal tissue area was covered by Dox administered in isotonic solution, whereas $88 \pm 6\%$ was covered when administered in hypotonic solution (Fig 1A). When the mice were ambulatory for 10 mins prior to tissue collection, the isotonic solution delivered Dox to $23 \pm 3\%$ of the vaginal surface area, whereas the hypotonic solution delivered it to $86 \pm 6\%$ of the area (Fig 1B). The isotonic solution only delivered drug to the vaginal surfaces facing the lumen, not the surfaces contained within the collapsed vaginal folds, thus producing the pattern of "stripes" on the flattened tissue with the unexposed tissue appearing black (Fig 1C). In contrast, drug administered in hypotonic solution was distributed to the entire vaginal surface (Fig 1D).

Effect of tonicity on vaginal drug retention

We next investigated whether hypotonic fluid absorption would also lead to more rapid absorption of the drug across the epithelium, thereby potentially reducing vaginal retention.

Isotonic and hypotonic Dox solutions were administered intravaginally to anesthetized mice to avoid any effect of solution leakage. After 10 min in the supine position, the entire reproductive tract was excised and analyzed quantitatively with fluorescence imaging (Fig 2A). After only 10 min, the relative fluorescent signal of Doxorubicin administered in hypotonic solution was half that of Doxorubicin administered in isotonic solution (Fig 2B).

Effect of tonicity on prevention of vaginal HSV-2 infection

We then tested whether the enhanced distribution of hypotonically administered drug at short times, and the reduced retention at longer times, would affect the efficacy of a vaginally administered drug. We chose to use acyclovir monophosphate (ACVp), a moderately protective drug that we previously demonstrated provided partial protection when administered 30 min prior to viral inoculum in a mouse model of vaginal HSV-2 infection [16], since it would likely reveal any benefit of improved drug distribution. Similar to Dox, ACVp is water soluble and its mechanism of action is intracellular. First, we ensured that hypotonic fluid administration did not increase susceptibility to infection by administering the ID₅₀ dose of virus (typically infects ~50% of the mice) suspended in either isotonic or hypotonic solution. When virus was administered in either isotonic or hypotonic solution, 60% (9/15) mice were infected (data not shown). This control experiment indicated we could consider changes in infection rate to be due to the presence of the drug and not from hypotonic effects on the virus or tissue susceptibility. For protection studies, we then used 10ID₅₀, a dose that typically infects ~90% of the mice. When 1% ACVp was administered 1 min before virus, 49% (22/45) of mice were infected when ACVp was administered isotonic, and 31% (14/45) of mice were infected when ACVp was administered hypotonically (Fig 3). Although this result suggested the hypotonic solution may have increased vaginal protection, this difference was not statistically significant with the group size studied ($p = 0.1$). When 1% ACVp was administered 1 h before virus, 45% (27/60) of mice were infected when ACVp was administered isotonic, and 73% (33/45) of mice were infected when ACVp was administered hypotonically (Fig 3). This difference was statistically significant ($p < 0.01$), suggesting that although hypotonic delivery may have improved immediate protection, the osmotic flow through the epithelium appeared to remove unencapsulated ACVp from the vagina by rapid absorption, leading to decreased protection.

Effect of osmotically-driven convection on vaginal nanoparticle distribution

Hypotonic delivery of free drug led to improved distribution in the vaginal tract, but osmotically-induced absorption of fluid may cause drugs for which the vaginal epithelium is permeable to be removed by rapid absorption. In contrast, an intact vaginal epithelium is essentially impermeable to nanoparticles, and, if the particles are mucus-penetrating, they can be rapidly carried by osmotic flow to the epithelial surface. We have previously demonstrated that MPP, capable of penetrating vaginal mucus, coat the entire vaginal surface within 10 min after being delivered in a hypotonic solution [16]. Conversely, even if delivered in hypotonic fluid, mucoadhesive conventional nanoparticles (CP) aggregated in the luminal mucus layers and are not transported through vaginal mucus to the vaginal epithelium by osmotically-induced convection [16]. Here, we investigated the dependence on tonicity of osmotically-driven distribution of MPP immediately after administration. By freezing vaginal tissues immediately after MPP administration, we took a “snap-shot” of the initial particle distribution. When MPP were administered in isotonic solution, the nanoparticles were found distributed throughout the vaginal lumen (Fig 4), but when delivered in hypotonic solutions, a gradient in particle concentration was apparent, with MPP being concentrated at the surface of the vaginal epithelium (Fig 4). From the surface distribution of MPP as shown in Figure 4, it is evident that hypotonically-induced fluid flow

resulted in MPP being rapidly drawn to the vaginal surface, within minutes, without being transported across the epithelium.

Effect of tonicity on vaginal nanoparticle retention

We then hypothesized that, in contrast to free drug administered in hypotonic solution, vaginal retention of MPP would improve with hypotonic delivery. Since we anticipated that fluid leakage would play a role in nanoparticle retention, we compared both non-ambulatory and ambulatory conditions. In the case of non-ambulatory mice (supine for 1 h), $69 \pm 17\%$ of MPP administered in isotonic solution and $83 \pm 11\%$ of MPP administered in hypotonic solution were retained (Fig 5A). It is likely that the 1 h period allowed for fluid absorption and removed gravitational effects, reducing leakage. Although a higher percentage of MPP were retained when administered in hypotonic solution, the difference was not statistically significant. However, when the mice were ambulatory, there was a significant decrease in the retention of MPP administered in isotonic solution. After 10 min of ambulation, only $22 \pm 10\%$ of MPP administered in isotonic solution were retained compared with $75 \pm 19\%$ of MPP administered in hypotonic solution (Fig 5B). It appears that the rapid delivery of MPP to the vaginal surface led to the increased retention since much of the fluid in the vagina is discharged during ambulation [25].

Effect of osmolality on vaginal nanoparticle distribution

It is well known that cells are sensitive to the tonicity of their surroundings, and recent evidence highlights the toxicity of strongly hypertonic gels on both vaginal and rectal epithelia, particularly with repeated exposure [2, 16]. To avoid potential toxic effects, we investigated whether modest levels of hypotonicity might still improve particle distribution in the vagina. Within 10 min of administration, MPP in hypotonic solutions covering a 10-fold range (20-220 mOsm/kg) delivered MPP to the vaginal surface (Fig 6A). In contrast to an isotonic solution (294 mOsm/kg) that left MPP particles distributed throughout the vaginal lumen with few being drawn to the epithelial surface, a modestly hypotonic solution (220 mOsm/kg) caused rapid transport to the epithelial surface (Fig 6A). Also, there was a trend toward increased vaginal coverage with increasing hypotonicity: in the most hypotonic solution (20 mOsm/kg), MPP coated $88 \pm 2\%$ of the vaginal epithelium after 10 min [16], whereas only $60 \pm 4\%$ of the vaginal surface was coated with MPP administered in 294 mOsm/kg solution (Fig 6B). At all tonicities tested, MPP reached a greater fraction of the epithelial surface than muco-adhesive (CP) particles, and the minimally hypotonic solution (220 mOsm/kg) delivered particles to $76 \pm 2\%$ of the vaginal surface, a significant increase compared with $60 \pm 4\%$ coverage by the isotonic solution (294 mOsm/kg).

Discussion

Poor drug distribution significantly limits effective drug delivery in the vagina [26, 27]. Poor drug distribution into the rugae, even after simulated intercourse, has been cited as a critical factor in the failure of microbicide products to protect against vaginal infection [12]. Other microbicide studies in mice have used large volumes of test product (up to 40 μ l) [28, 29], to promote more complete vaginal distribution [30]. In our experience, the mouse vagina can hold a volume of ~ 50 μ l; such a comparatively large volume of test agent distends and unfolds the vaginal epithelium. In contrast, the human vagina can retain in the range of 50 ml [31], while typical vaginal products deliver only 2-5 ml [26, 27]. Here, to investigate vaginal distribution in mice *in vivo*, we used a small volume (5 μ l) that would more appropriately mimic the volumes used in humans. We demonstrated a simple method that delivers drugs into the deep folded surfaces, without distending the vagina, which is expected to lead to more effective vaginal drug delivery.

Vaginal drug delivery as a means for achieving both local and systemic delivery has received considerable attention recently [32, 33]. The vaginal epithelium is permeable to small molecules, and is, therefore, capable of absorbing various drugs [33]. The superficial layer of the human vaginal epithelium contains densely packed dead and dying cells (the stratum corneum) that protects the deeper living cell layers while allowing secretion and absorption of fluid through the epithelium [34]. In women with healthy vaginal microbiota (lactobacilli), total vaginal secretion (mostly from the endocervix and upper vagina) was measured at ~6 g/day, while ~4 g/day was reabsorbed by the lower introital vagina [35]. Thus, the vagina has a native capacity for osmotically-induced fluid absorption that can be used for drug delivery. We hypothesized that absorption of hypotonic fluids containing drugs would result in distribution throughout the entire vaginal epithelial surface. Indeed, Dox administered in hypotonic solution coated more than 85% of the vaginal surface of ambulatory mice, whereas only 23% of the vaginal tissue surface was coated with Dox administered in isotonic solution. The isotonic fluid does not penetrate into the rugae, leaving a striping pattern when the vaginal tissue is opened and flattened. Others have suggested that hypotonic delivery could increase the contraceptive efficacy of nonoxynol-9 (N9) by improving mobility of N9 through mucus to reach sperm in the mucus [36, 37].

Although there was improved distribution of drug in hypotonic solution, fluid absorption could potentially lead to rapid removal of the drug by fluid transport through the vaginal epithelium. Rapid drug absorption with hypotonic administration is likely an effective means of reaching systemic circulation, but not for delivering drug locally in the vagina. Using acyclovir monophosphate (ACVp), we demonstrated a trend toward improved protection by ACVp in hypotonic solution vaginally administered immediately before HSV-2 virus. The improved protection is likely due to the increase in vaginal coverage by drug administered in hypotonic solution. In contrast, when HSV-2 was administered 1 h after the drug, the protection by ACVp administered in hypotonic solution was significantly decreased compared to ACVp in isotonic solution. Drug absorption across the epithelium with osmotically-induced fluid flow likely led to drug clearance and decreased protection after 1 h. MPP offer a way to achieve improved distribution of drug by hypotonic delivery since nanoparticles accumulate on the surface of the vaginal epithelium, where they then slowly release drug. As a result, we demonstrated that mucus-penetrating nanoparticles (MPP) containing ACVp demonstrated better protection than 10-fold higher concentration of free drug when administered vaginally 30 min prior to HSV-2 virus [16]. Here, we showed that MPP are immediately drawn to the vaginal epithelium when administered in a minimally hypotonic solution compared to administration in an isotonic solution.

Fluid absorption and secretion can also affect vaginal retention due to product leakage. It was previously demonstrated in humans that the leakage rate of vaginal gels increased linearly with increasing hypertonicity [1]. In this case, retention was reduced by osmotically-induced fluid *secretion* leading to product leakage. This of course suggests that hypotonic products would cause fluid absorption and reduced leakage, and might thereby improve vaginal retention. We found that this was true for MPP, but not necessarily true for unencapsulated drugs that can be absorbed through the vaginal epithelium. We previously demonstrated that by penetrating into the more slowly cleared mucus layers in the deep vaginal folds, MPP are better retained (60% after 6 h) than mucoadhesive CP (10%) [16]. Here, we demonstrated that although MPP are retained similarly in non-ambulatory, supine mice (eliminating leakage due to gravitational and peristaltic effects) when administered in hypotonic or isotonic solution, MPP are better retained in the vaginal tract of ambulatory mice when administered in rapidly-absorbed hypotonic solution (75% after 10 min) as compared to administration in isotonic solution (22%). Our results indicate that a hypotonic gel formulation containing MPP would likely enhance both drug distribution and retention in the human vagina.

We anticipate that the benefits of hypotonic delivery described here are broadly applicable to many types of MPP with mucoinert surface coatings. Generally, advective delivery through mucus to the epithelium is only possible for nanoparticles that can penetrate through, rather than become adhesively trapped in, mucus. We previously demonstrated that MPP were transported along with flowing fluid through human CVM, while mucoadhesive CP remained stuck in place; only after removing the vaginal mucus by lavage were CP able to distribute throughout the mouse vagina similar to MPP [16]. Also, MPP with a biodegradable core distributed similarly to non-biodegradable MPP in the mouse vagina [16]. Thus, the surface characteristics (and therefore potential for adhesive interactions with the superficial mucus barrier layers), rather than the core nanoparticle composition, are important. We have also recently demonstrated that nanoparticle interactions with mucus observed by multiple particle tracking (MPT) are directly correlated with distribution *in vivo*. MPP that diffused rapidly in mouse vaginal mucus, as observed by MPT, distributed throughout the entire vagina when administered hypotonically, lining the epithelium and the deep folds. In contrast, particles larger than the mucus pores or particles with mucoadhesive surfaces were immobilized or exhibited significantly hindered diffusion in mucus when observed by MPT, and this correlated with trapping and poor distribution in the superficial luminal mucus layers *in vivo*, where the particles were kept away from the vaginal epithelium [38]. Thus, particles unable to penetrate mucus, either due to adhesive or steric exclusion, were not effectively delivered to mucosal epithelia by osmotically-induced advection. In contrast, particles that can rapidly diffuse through mucus, as observed by MPT, distribute rapidly and evenly over mucosal epithelia when administered hypotonically.

Most vaginal gels have been formulated with excipients such as glycerin or propylene glycol, which render the gels hypertonic. Unfortunately, recent investigations show that these hypertonic formulations cause toxicity in the vaginal tract of mice that increases susceptibility to HSV-2 infection [3], which is likely a result of their hypertonicity [2, 39]. In addition a hypertonic gel formulation was found to disrupt epithelial integrity in the human colon [2], and a hypertonic tenofovir gel formulation was found to induce epithelial fracture in ectocervical and colorectal explants compared with tissues exposed to medium only [4]. It has been postulated that a major contributing factor in the dextran sodium sulfate (DSS)-induced experimental irritable bowel disease mouse model is the hypertonicity of the DSS solution [40, 41]. In previous work, we observed an increase in inflammatory cytokine release in mouse vaginal lavage fluid after 7 consecutive daily doses of a hypertonic gel vehicle, but no increase after 7 consecutive daily doses of a hypotonic formulation [16]. It is not yet known whether purely hypotonic delivery vehicles will have toxic effects after prolonged, repetitive exposure. Hypotonic stress has been used in cell culture as a means to increase viral uptake for replication; cells were exposed continuously for 4 h and yet had no reduction in viability [42]. Hypotonic solutions did not increase susceptibility to vaginal HSV-2 infection. However, this does not rule out potential for toxic effects after repeated hypotonic shocks, which led us to investigate whether minimally hypotonic formulations could still improve MPP distribution. Indeed, we found that a reduction in osmolality from 294 mOsm/kg to 220 mOsm/kg increased vaginal surface coverage from 60% to 76%, and essentially all hypotonically delivered MPP were drawn from the lumen to reach the epithelial surface deep within folded surfaces within 10 min of administration.

Conclusion

We investigated the use of hypotonic solutions for convection-enhanced drug delivery to the vagina. We found that although hypotonic delivery of Doxorubicin improved the vaginal distribution, the drug was rapidly absorbed through the epithelium when dosed this way, reducing vaginal retention. In contrast, we found that hypotonic delivery of mucoinert mucus-penetrating nanoparticles (MPP) improved both distribution and retention. In

addition, we found that even slightly hypotonic delivery significantly improved the vaginal distribution of MPP. Our results indicate that hypotonic formulations may be more effective for drug delivery to the vagina than the traditional hypertonic formulations, and that MPP delivered hypotonically offer significant promise for non-toxic sustained drug delivery to the entire vaginal surface.

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Figure 1. Vaginal coverage by Doxorubicin (Dox) administered in either hypotonic (hypo) or isotonic (iso) solution

Mice (A) remained supine for 1 hr prior to tissue collection (non-ambulatory) or (B) were ambulatory for 10 minutes prior to tissue collection (ambulatory). Images representative of the average vaginal surface coverage for ambulatory mice dosed with Dox in (C) isotonic (iso) and (D) hypotonic (hypo) solution. Data are means $\pm$ SEM ($n = 5$). * $P < 0.05$ compared to isotonic, Wilcoxon rank-sum test.

Figure 2. Vaginal retention of Doxorubicin administered in either isotonic (iso) or hypotonic (hypo) solution

Mice remained supine for 10 minutes prior to tissue collection. Overlay of Doxorubicin fluorescence intensity and bright-field images for (A) isotonic solution and (B) hypotonic solution in whole cervicovaginal tract tissue. (C) Relative Doxorubicin signal based on quantification of fluorescent signal, adjusted for solution fluorescence. Images are representative of the averages calculated for $n = 4$ mice and were quantified as relative signal $\pm$ SEM. * $P < 0.05$ compared to isotonic, Wilcoxon rank-sum test.

Figure 3. Vaginal HSV-2 infection after treatment with acyclovir monophosphate (ACVp) in either hypotonic (hypo) or isotonic (iso) solution
ACVp (10 mg/ml) was administered 1 min or 60 min prior to viral inoculum. $n = 45$ mice were tested in each group, and infection rates in the control group were ~90%. * $P < 0.05$ compared to isotonic, Fisher's exact test.

Figure 4. Vaginal distribution of 100 nm fluorescent MPP administered in isotonic (iso) or hypotonic (hypo) solution

MPP distribution in transverse mouse vaginal cryosections. Tissues were collected and frozen immediately after particle administration. Images are representative of $n = 3$ mice. Cell nuclei stained blue with DAPI.

Figure 5. Vaginal retention of MPP administered in either hypotonic (hypo) or isotonic (iso) solution

(A) Mice remained supine for 1 hr prior to tissue collection (non-ambulatory). (B) Mice were ambulatory for 10 minutes prior to tissue collection (ambulatory). Particle retention was calculated as mean $\pm$ SEM ($n = 5$). * $P < 0.05$ as compared to isotonic, Wilcoxon rank-sum test.

Figure 6. Vaginal distribution of fluorescent 100 nm MPPs administered in solutions of varying osmolality

(A) Distribution of MPP in transverse vaginal cryosections. (B) Quantified surface coverage of MPP on whole, flattened vaginal tissue. All tissues were collected within 10 min of particle administration. All values have units mOsm/kg. Osmolality for mucoadhesive CP particles was 20 mOsm/kg. Images are representative of $n = 5$ mice. Data calculated as means $\pm$ SEM ($n = 3$). #Reprinted from (16). * $P < 0.05$ as compared to hypotonic solutions (20-220), Wilcoxon rank-sum test.