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Development of a mucoinert progesterone nanosuspension for safer and more effective prevention of preterm birth

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ABSTRACT

Preterm birth (PTB) is a significant global problem, but few therapeutic options exist. Vaginal progesterone supplementation has been demonstrated to reduce PTB rates in women with a sonographic short cervix, yet there has been little investigation into the most effective dose or delivery form. Further, vaginal products like progesterone gel often contain excipients that cause local toxicity, irritation, and leakage. Here, we describe the development and characterization of a mucoinert vaginal progesterone nanosuspension formulation for improved drug delivery to the female reproductive tract. We compare the pharmacokinetics and pharmacodynamics to the clinical comparator progesterone gel in pregnant mice and demonstrate increased vaginal absorption and biodistribution via the uterine first-pass effect. Importantly, the unique plasma progesterone double peak observed in humans, reflecting recirculation from the uterus, was also observed in pregnant mice with vaginal dosing. We adapted a mouse model of progesterone withdrawal that was previously believed to be incompatible with testing the efficacy of exogenous progestins, and are first to demonstrate efficacy in preventing preterm birth with vaginal progesterone in this model. Further, improved vaginal progesterone delivery by the nanosuspension led to increased efficacy in PTB prevention. Additionally, we identified histological and transcriptional evidence of cervical and uterine toxicity with a single vaginal administration of the clinical gel that are absent after dosing with the mucoinert nanosuspension formulation. We demonstrate that a progesterone formulation that is designed for improved vaginal progesterone absorption and vaginal biocompatibility could be more effective for PTB prevention.

1. Introduction

Preterm birth (PTB) remains a significant global problem with an estimated 15 million babies born preterm a year [1]. PTB is defined as delivery before 37 weeks gestation and remains the highest contributor to neonatal mortality and morbidity. By a 2006 estimate, the yearly (US) health care cost associated with taking care of preterm babies was a staggering $26 billion [2]. This does not account for the life-long heath care costs associated with the adverse health consequences that preterm babies face, including but not limited to, respiratory distress syndrome, neurodevelopmental disability, cerebral palsy, retinopathy of prematurity, and necrotizing enterocolitis [3,4]. The causes of PTB are multifactorial, and only a few clinically proven interventions exist for preventing non-medically indicated PTB [5]. The only therapeutic moiety that has shown efficacy in PTB prevention is progesterone, which is also known as “the pregnancy hormone” [6]. Natural

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progesterone plays essential roles in establishment and maintenance of pregnancy. As such, vaginal administration of natural progesterone has been shown to both support the success of in vitro fertilization (IVF) [7] and prevent preterm birth in women with signs of premature cervical remodeling (sonographic short cervix) [8]. However, there has been very little investigation into the most effective dose or delivery form for natural progesterone [9].

The vaginal route of drug administration has many advantages, including avoidance of hepatic first-pass and allowance of using lower drug doses [10]. Furthermore, vaginally administered drugs can take advantage of the "uterine first-pass effect", a phenomenon in which drugs are transported through counter current exchange between the vaginal/uterine venous, arterial and lymphatic networks. By utilizing the uterine first-pass effect, drugs can be preferentially delivered to the upper reproductive tract without first being absorbed and diluted into the systemic circulation [11]. Thus, the vaginal route of administration is particularly attractive for women's health applications, including fertility assistance and PTB prevention. Crinone® micronized progesterone gel is approved for vaginal administration for assisted reproductive technology and secondary amenorrhea. Crinone® (8%) has also been used in clinical studies for preterm birth prevention [12–14]. However, some women complain of vaginal irritation and vaginal discharge [5,15], likely due to the high osmolality. Hypertonic vaginal formulations have been shown to cause epithelial toxicity and vaginal leakage [16–18]. Indeed, it is often the case that vaginal products are not optimized for vaginal drug absorption, retention, and safety [16,18–20], which can limit efficacy.

The application of nanomedicine has revolutionized mucosal drug delivery, including the respiratory tract, gastrointestinal tract, ocular surface, bladder, and female reproductive tract [20–26]. Mucus acts as a primary barrier to drug absorption and retention, and the physiology and structure of the various epithelial surfaces further limit effective drug delivery. In the vagina, drug distribution and retention are limited by the cervicovaginal mucus (CVM), the highly folded epithelial surface, intra-abdominal pressure, gravitational forces, and osmotic forces [19,20]. We have demonstrated that water absorption induced by hypotonic vehicles increases the vaginal distribution of nanoparticles coated to be non-adhesive to mucus (mucoinert) [20]. We have also observed that mucoinert nanosuspensions can enhance mucosal absorption of poorly soluble drugs by both bypassing the mucus barrier and increasing the surface area for dissolution and absorption [27]. Thus, we hypothesized that vaginal administration of a mucoinert progesterone nanosuspension (NS) in a hypotonic vehicle could lead to enhanced vaginal absorption of progesterone compared to the hypertonic, micronized progesterone gel (Gel). There is potential that increased vaginal progesterone absorption and distribution to the upper female reproductive tract could lead to improved prevention of PTB.

Here, we describe the formulation of a hypotonic, mucoinert progesterone NS. We first compared the pharmacokinetics of the progesterone NS and Gel after vaginal administration to pregnant mice. We further compare the efficacy for PTB prevention in a mouse model of progesterone withdrawal (mifepristone, or RU486). To our knowledge, this is the first report of any vaginal progesterone formulation that successfully prevented preterm birth in a preclinical animal model. The ability to test formulations in preclinical animal models enables optimization of the pharmacokinetics and pharmacodynamics. Further, we found that the progesterone NS provided a significant improvement in PTB prevention compared to Gel, while also appearing to have improved biocompatibility for vaginal administration. The combination of more effective vaginal drug delivery and enhanced biocompatibility has potential to improve therapeutic interventions for preventing PTB.

2. Materials and methods

2.1. Probe nanoparticle formulation and mucus tracking

Polyethylene glycol (PEG)-coated nanoparticles were prepared as previously described [28]. Briefly, 200 and 500 nm carboxylate-modified polystyrene (PS) beads (Molecular Probes) were coated with 5 kDa methoxy- PEG-amine (Creative PEGworks) using 200 mM borate buffer (pH 7.4). For nanoparticles with adsorbed polymer coatings, PS beads (200 nm and 2 μm) were diluted 10-fold with 2% (*w/v*) Pluronic F127 solution in water or with 0.5% (*w/v*) polycarbophil (Lubrizol NOV1001) solution in water. Particle solutions were incubated at 4 °C overnight. A 2.5 mg/mL curcumin (Sigma C1386) nanosuspension was formulated via wet milling using a bead-based tissue homogenizer, similar to the optimized progesterone conditions described below, for 10 h. Multiple particle tracking experiments were performed using freshly obtained, undiluted CVM from pregnant women. Participant and sample characteristics are outlined in Supplementary Tables 1 and 2. All CVM samples used were "healthy" according to a subset of Amsel's criteria, including pH < 4.5, absence of "clue" cells in the wet mount, and negative "whiff" test. CVM was self-collected as previously described [29] and samples were stored at 4 °C and used within 24 h of collection. PS and PS-PEG nanoparticles were diluted to 0.1% *w/v*, and 0.5 μL was added to 20 μL of CVM in a custom made sample well that was immediately sealed with a coverslip. For CVM samples with low volume, a 5 μL well was used, with 0.4 μL of particles added. Twenty second videos of nanoparticle diffusion in CVM were recorded at room temperature using a Zeiss Axio Observer inverted epifluorescence microscope equipped with a 100×/1.46 NA oil-immersion objective and an EM-CCD camera (Evolve 512; Photometrics) with image resolution of 25 nm/pixel and at a frame rate of 15 Hz (34 Hz for 2 μm particles due to increased fluorescence intensity). For each CVM sample, 3–5 videos were collected. Nanoparticle trajectories were analyzed using automated MATLAB-based particle tracking software with a minimum of 16 frames (~1 s) of consecutive tracking.

2.2. Nanosuspension formulation and characterization

Crinone® 8% gel (Actavis Pharma, Inc.) was sourced from Johns Hopkins Pharmacy. Progesterone (P8783-5G) was sourced from Sigma-Aldrich and Pluronic® F127 was generously provided by BASF (Ludwigshafen, Germany). Initial formulation work was conducted at 10 mg/mL progesterone and included both top-down (high pressure homogenization & wet milling) and bottom-up (nanoprecipitation) approaches. High-pressure homogenization (HPH) was done with 10 cycles through Avestin EmulsiFlex B15 (Ottawa, ON) at 60 psi for 10 mg/mL progesterone in 2% F127. For nanoprecipitation (NP), 10 mg/mL progesterone and 2% F127 was dissolved in acetone and added dropwise to a solution of water with mechanical stirring. The solution was stirred in a fume hood for up to 4 h to evaporate the acetone. However, we found these techniques did not provide sufficient size reduction and/or resulted in higher than desired heterogeneity (see Fig. 1a). In contrast, we found that wet-milling using a bead-based tissue homogenizer TissueLyser LT (Qiagen) with 0.5 mm zirconium oxide beads (Next Advance) at ~1.5 g per mL of 10 mg/mL and 2.4 g per mL of 80 mg/mL progesterone was more effective in reducing the particle size to the target size range. Initial optimizations included varying the concentration of Pluronic F127 from 0.5–4% (*w/v*) and milling for various times up to 9 h. Wet milling was done in a cold room to mitigate heat generation during the extended milling times. Particle size, polydispersity index (PDI), and surface charge (ζ-potential) of progesterone nanosuspension (NS) formulations were measured using a Malvern Zetasizer Nano ZS (173° scattering angle). NS were diluted 1:1000 in 10 mM NaCl (pH 7) for ζ-potential measurements. The final NS formulation contained 80 mg/ml progesterone (8%) in 2% F127 and each batch was milled for ~8–10 h until the final average size of

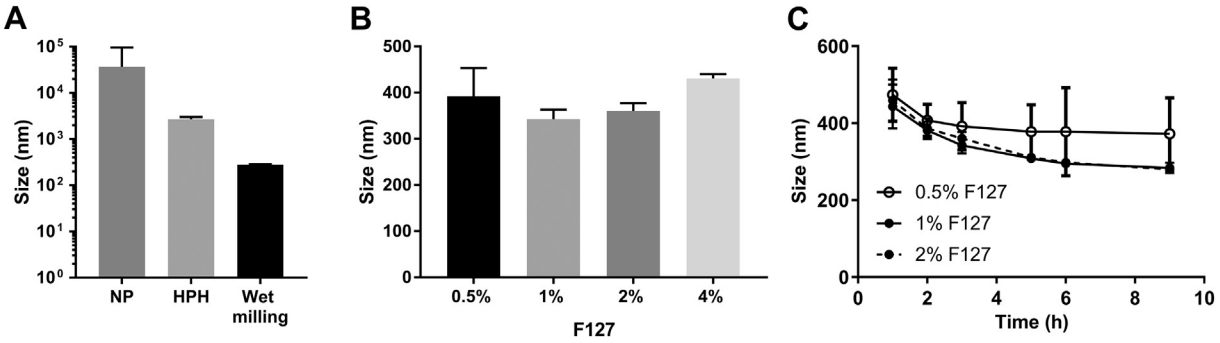


Fig. 1. Progesterone particle size obtained as a function of (A) size reduction technique (wet milling time 9 h), (B) concentration (*w/v*) of Pluronic F127 in the supernatant during milling optimization, and (C) milling time for batches with different F127 concentrations. Data are represented as average ± SEM for $n = 3$–4 batches per group. NP = Nanoprecipitation, HPH = high pressure homogenization.

~260 nm was obtained (Supplementary Table 3). Osmolality measurements were conducted using a Vapro vapor pressure osmometer (model 5600). Osmolality of NS was measured without dilution. Because of the high gel viscosity and high osmolality values falling outside the linear measurement range of the instrument, the Gel was diluted 10-fold with ultrapure water and the measured osmolality values were multiplied by 10.

2.3. Animals

Timed pregnant 6–8 weeks old female CD-1 mice were ordered from Charles River Laboratories (Wilmington, MA). Pregnant mice were delivered to the animal facility about E8-E10 and allowed to acclimate in a reverse light cycle room (dark period 10 am–10 pm) at Johns Hopkins University prior to procedures. Procedures took place on day 15 of gestation (E15) out of a total gestation length of about 19.6 (average; range 19–20 days), unless otherwise specified. The guaranteed pregnancy success rate for timed pregnant animals was ≥70%, and only mice that were pregnant by visual observation were included in experiments.

2.4. Ethical statement

All animal procedures were approved by the Johns Hopkins University Animal Care and Use Committee. Procedures for CVM self-collection were approved by the Johns Hopkins University School of Medicine Institutional Review Board under IRB studies NA_00038105, NA_00085130, and IRB00099798. Informed consent was obtained from all human subjects.

2.5. RU486 model of preterm birth

RU486 (Sigma-Aldrich M8046) progesterone antagonist was used to induce preterm birth in mice. RU486 was dissolved in dimethylsulfoxide (Sigma-Aldrich) at a concentration of 0.25–1.5 mg/mL. Pregnant mice received a subcutaneous injection in the scruff of the neck containing 25–150 μg RU486 in 100 μL on the morning of E15. We observed that the 25 μg dose was sufficient to cause 85% preterm birth, defined as delivery of at least one fetus within 24 h of injection (Supplementary Fig. 1). Vaginal progesterone treatment studies were conducted with the 25 μg RU486 dose. Mice receiving vaginal progesterone treatment were given 8 mg of progesterone using a 50 μL Wiretrol (Drummond Scientific) in the form of either 100 μL of gel (Crinone®) or 100 μL of the NS formulation. Mice receiving vehicle control were given 100 μL of 2% F127. NS formulations were prepared fresh daily up to 14 h prior to animal dosing with size and ζ-potential measurements done just prior to dosing. Treatment began at the time of RU486 injection and continued daily in the morning through E18 for a total of four vaginal doses. Mice did not receive additional treatment if they delivered preterm. Animals were counted as delivery at term if delivery happened on or after the morning of E19 and resulted in live births. Efficacy experiment data (Fig. 4) was pooled from 4 identically designed replicate experiments on 4 different days with $n = 5$ pregnant mice in each group. One replicate had only $n = 4$ for the RU486 + Gel dosing arm, resulting in $n = 19$ for that group. A RU486 control group was run in parallel to treatment arms for each replicate experiment. Induction experiments with 150 μg of RU486 was one replicate, while 50 μg RU486 was pooled from 3 replicate experiments conducted on 3 different days. The vehicle control experiment included $n = 5$ mice. No predetermined statistical method was used to calculate the sample size for the efficacy experiment, as there has been no prior example of progesterone dosing successfully preventing PTB in the RU486 model. No randomization was employed aside from random selection of animals shipped together as a group to assign to different treatment groups prior to dosing. Due to differences in rheological characteristics and appearance of the progesterone formulations, treatment allocation was not performed in a blinded manner. However, different study personnel were responsible for dosing and checking the cages for signs of labor/delivery.

2.6. Pharmacokinetics

Healthy pregnant mice were dosed vaginally with either 8 mg of Crinone® progesterone gel or 8 mg of progesterone NS. Mice were put into groups ($n = 4$–5 per group) and were sacrificed at 0.5, 1, 2, 3, 6 and 24 h. Plasma, cervix, proximal and distal uterus, and vaginal tissue were collected. To remove luminal Gel or NS from the ectocervix, we immediately embedded the cervix in Tissue-Tek® optimum cutting temperature (O.C.T) compound (Sakura Finetek USA, Inc.). The superficial ~100–140 μm of tissue was removed by cryosectioning using a Leica CM3050S Cryostat. The O.C.T was then thawed to remove the cervix tissue, and the tissue was rinsed with 10 mL of DPBS prior to being flash frozen with liquid nitrogen. In contrast to the rigid, relatively flat, and relatively small cervix tissue, we could not take the same sectioning approach to remove excess product from the thin and foldable vaginal tissue surface. To remove luminal excess Gel or NS from vaginal tissue, freshly excised vaginal tissue was bisected to expose the luminal surface before vortexing in 10 mL of 1% Tween 80 in DPBS for 60 s. The vaginal tissue was briefly dried by placing on a Kimwipe. Samples were flash frozen and submitted to the Johns Hopkins Analytical Pharmacology Core for progesterone detection. Progesterone was quantified in charcoal stripped (2×) mouse EDTA plasma (BioIVT) and cervical and vaginal tissue. Tissue samples were homogenized in 200 μL or 400 μL of blank charcoal stripped mouse plasma using Ultra Turrax T25 homogenizer (IKA) before extraction. Progesterone was extracted from 50 μL of plasma or tissue homogenates with 0.5 mL of acetonitrile/n-butyl chloride (1:4, *v*/v) containing 40 ng/mL of the internal standard, progesterone-d9 (Toronto Research Chemicals). After

centrifugation, the top layer was then transferred to a clean glass tube and dried in a 40 °C water bath under a stream of nitrogen gas. The samples were reconstituted with 100 μL of water/acetonitrile (50:50, *v/v*) and then transferred into autosampler vials for LC-MS/MS analysis. Separation was achieved with an Agilent Zorbax XDB, C18 (4.6 × 50 mm, 5 μm) column at room temperature with water/acetonitrile/formic acid mobile phase (30:70:0.1, v/v) using isocratic flow at 1 mL/min for a total of 4 min. The column effluent was monitored using a Sciex triple quadrapole™ 5500 mass-spectrometric detector (Sciex) using electrospray ionization operating in positive mode. The spectrometer was programmed to monitor the following MRM transition 315.3 → 109.1 for progesterone and 324.3 → 100.0 for the internal standard, progesterone-d9. Calibration curves for progesterone were computed using the area ratio peak of the analysis to the internal standard by using a quadratic equation with a $1/x^2$ weighting function over the range of 2 to 2000 ng/mL with dilutions up to 1:1000 (v:v). Tissue samples were then quantitated in ng/g as: nominal concentration (ng/mL) x dilution factor. Area under the curve from $t = 0$ to $t = 6$ h ($AUC_{0–6}$) or the last measured concentration (AUC_{last}) were calculated using sparse sampling noncompartmental analysis in Phoenix 64 WinNonlin® software.

2.7. Tissue collection and staining

Cervices used for gene expression were harvested by dissection approximately 16 h after RU486 injection. E15 control group tissue was dissected at the same time as RU486 and treatment groups. A dissecting microscope was used to ensure the complete removal of vaginal and uterine tissue. Myometrium tissue was harvested from a single uterine horn and decidua was scraped off using mechanical action with 0.009″ disposal razor blades (Personna American Safety Razor Co.). Samples for RNA isolation and quantitative real-time PCR were weighed and flash-frozen immediately after collection. Both tissue types were homogenized using an IKA T10 basic with a S10 N-10G-ST dispersion element. For staining, samples were processed, embedded, and stained by the Johns Hopkins Reference Histology Laboratory. Cervices were dissected 12 h after RU486 injection using dissecting microscope and fixed with formalin solution (10% neutral buffered, Sigma-Aldrich). Sections were embedded in paraffin and sectioned longitudinally (4 μm thick) and stained with either Masson's Trichrome (collagen density), hematoxylin and eosin (H&E, tissue morphology) or mucicarmine (cervical mucus production). Representative images of the Masson's Trichrome stained slides were obtained at 10× magnification using a Nikon Eclipse N*i*−U microscope equipped with a Nikon Fi3 color camera. Digital images of H&E and mucicarmine stained slides were captured at 400× magnification with an Olympus BX43 microscope equipped with a digital camera (DP26, Olympus). An ACVP certified veterinary pathologist examined all slides in a blinded fashion.

2.8. RNA Isolation and quantitative real-time PCR (qRT-PCR)

Cervices and myometrial tissue were processed for RNA isolation. The RNeasy Mini Kit (Qiagen) was used to extract RNA per manufacturer's instructions. Myometrial tissue was stored in 1 mL of RNA*later* (Invitrogen™) which was removed prior to homogenization. For myometrial tissue, 1 mL of TRIzol™ (ThermoFisher) was added to the sample and homogenized as described earlier. Fully homogenized samples were centrifuged at 21,130 rcf for 5 min at 4 °C. 700 μL of the supernatant was further processed through RNeasy mini kit. The RNA concentration was determined using a Thermo Scientific Nanodrop 2000 Spectrophotomer. Five micrograms of RNA were converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). Polymerase chain reaction (PCR) primers were synthesized using sequences listed in Supplementary Table 4. Each sample was run in triplicate for each primer. The 20 μL reaction included 3 μL of cDNA, 10 μL SYBR Green (ThermoFisher), 6 μL of water, and 0.5 μL of the forward primer at 5 pmol concentration and 0.5 μL of the reverse primer at 5 pmol concentration. qRT-PCR data were analyzed using the ΔΔCT method as described by user bulletin number 2 (Applied Biosystems). Reference genes *36b4* were used to normalize gene expression in the cervix, and the geometric mean of three previously described housekeeping genes, *Hprt*, *Ppia* and *Tbp* [30], was used to normalize expression in the myometrium.

2.9. Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used for comparing three or more groups in Graphpad Prism 7.03. Sample sizes were equal (or nearly so) in all experiments using ANOVA/Tukey. Log-rank test (Mantel-Cox) was done for all three curves in Graphpad Prism 7.03 ($p = .001$) testing the null hypothesis that all the samples come from populations with the same survival and that differences are due to chance. Multiple comparisons of the survival curves using pairwise comparisons between RU486 vs. Gel, RU486 vs. NS and Gel vs. NS was also done in Graphpad Prism 7.03. The statistically significant threshold for multiple comparisons was adjusted using the Bonferroni method (Bonferroni-corrected threshold $p = .017$). Log-rank p value was considered significant if $p < .017$. Pairwise comparisons of calculated AUC values were done using Bailer's method [31] which takes into account inter-animal variability and the pooled estimate of variance. Pharmacokinetic data was generally heterogeneous with high coefficient of variation (%CV) for all compartments and timepoints.

2.10. Data availability

Data are available upon request.

3. Results

3.1. Probe nanoparticle tracking in cervicovaginal mucus (CVM)

We have previously characterized the structural properties of cervicovaginal mucus (CVM) in healthy, nonpregnant women [32]. Because it is known that circulating hormone levels have an impact on mucus properties, we first aimed to characterize whether nanoparticles behave differently in CVM from pregnant women. We compared the mobility of both densely PEG-coated (PS-PEG) and uncoated (PS) polystyrene nanoparticles 200 and 500 nm in size. Similar to our prior work, we found that PS-PEG diffused over large distances in CVM from pregnant women, whereas uncoated PS nanoparticles were adhesively trapped (Supplementary Fig. 2). While the structural properties of mucus likely differ in the steroid hormone environment of pregnancy compared to nonpregnant, we found that the 200 and 500 nm PS-PEG nanoparticles were slowed only ~13 and ~16 fold, respectively, compared to what we previously observed in CVM from nonpregnant women [33]. We next compared the mobility of Pluronic F127 coated model particles to the PS-PEG particles. Because progesterone cannot be directly visualized for particle tracking, we used an autofluorescent drug with a similar log P and low water solubility, curcumin, to make a model NS (Supplementary Table 3). We also evaluated the coating used in Crinone to impart “bioadhesive” properties, polycarbophil, and larger model particles (2 μm PS) to mimic the Crinone particle size. PS particle coatings with F127 were evidenced by an increase in size and ζ-potential, while polycarbophil coatings led to an increase in size and a minor decrease in ζ-potential due to its polyanionic character (Supplementary Table 3). As shown in Supplementary Fig. 3a, we observed that 200 nm PS-PEG, 200 nm PS coated with 2% F127 (PS/F127), and an F127 coated curcumin NS (Curcumin NS/F127) ~260 nm in size diffused similarly rapidly in CVM from pregnant women, while 200 nm PS coated with polycarbophil were adhesively trapped in CVM (Supplementary Fig. 3a). In contrast, 2 μm particles were largely

immobilized in CVM, regardless of coating, reflecting steric trapping (Supplementary Fig. 3b). Thus, we determined that the progesterone nanosuspension should ideally be near 250 nm (actual size for 200 nm nanoparticles after coating) and an F127 coating to penetrate effectively through the CVM barrier during pregnancy.

3.2. Progesterone nanosuspension formulation

We used F127 as a stabilizer to formulate mucoinert progesterone nanosuspensions (NS) via nanoprecipitation, high pressure homogenization, and wet-ball nanomilling (Fig. 1a). We found that the particle size obtained via nanoprecipitation and high pressure homogenization were much larger than the target size, so we focused on the wet milling approach. Using wet milling, 1% and 2% F127 resulted in smaller average particle size (Fig. 1b) that reached a plateau after about 9 h of milling time (Fig. 1c). Because the target formulation concentration was 80 mg/mL progesterone to match the Gel, 2% F127 was chosen to balance achieving the minimum particle size with the maximal potential for colloidal stability. The progesterone concentration was increased from 10 to 80 mg/mL (8%) without a significant increase in particle size (not shown). The final progesterone NS size, ζ-potential, and osmolality are listed in Supplementary Table 3.

3.3. Pharmacokinetics of a single vaginal progesterone dose in pregnant mice

We next compared the pharmacokinetics of progesterone in relevant compartments after a single vaginal dose of either progesterone Gel or NS in healthy pregnant mice on gestation day 15 (E15). Because these formulations contain the natural form of progesterone and our analytical method cannot discern between exogenous and endogenous sources, we also measured the endogenous progesterone levels in a small subset ($n = 4$) of healthy untreated E15 mice (solid and dotted gray lines in Fig. 2). Frequent sampling was performed over the first 6 h to capture the rapid absorption and distribution of progesterone after vaginal dosing. Mice are known to have higher metabolic rates than humans [34], and thus, progesterone levels in all compartments characterized (plasma, cervix, and proximal and distal uterus), approached the median endogenous levels by 24 h after dosing (Fig. 2). According to the calculated PK parameters listed in Supplementary Table 5 and using the "rule of five" (approximates 4–5 half-lives for a drug to be completely cleared) [35], we can estimate that the majority of the exogenous progesterone was eliminated from the tissue compartments before 24 h (range 15.5–23.5 h). Thus, the endogenous progesterone levels constituted an increasingly large fraction of the progesterone exposure as time approached 24 h, and the areas under the curve (AUCs) were calculated for both 0➔6 h ($AUC_{0–6}$) and 0➔24 h (AUC_{last}). The p values for all pairwise comparisons of AUCs are shown in Supplementary Table 6.

In plasma, the progesterone concentrations were higher after NS dosing, with a notable double peak in absorption at 3 h (inset, Fig. 2), suggesting recirculation. Circulating progesterone levels were essentially baseline by the 6 h timepoint, and the $AUC_{0–6}$ for the NS was significantly (~2.3-fold higher, $p = .0004$) increased compared to Gel (Fig. 3). The NS also provided an increased plasma C_{max} (peak concentration) compared to Gel (Supplementary Table 5). The progesterone NS also provided increased absorption and exposure in the cervix (Fig. 2), with the $AUC_{0–6}$ being ~4.9-fold higher ($p = .03$) compared to Gel (Fig. 3). Furthermore, the C_{max} for the NS was ~7 fold higher than the Gel formulation in cervical tissue (Supplementary Table 5). Because the mouse uterus is bicornuate, we sampled both proximal (next to the cervix and closest to the administration site) and distal (next to the ovary) tissue. Interestingly, there was evidence of a second progesterone peak for both formulations 2 h after dosing in the distal uterus (Fig. 2), compared to the second peak at 3 h in plasma. The $AUC_{0–6}$ and AUC_{last} calculations trended toward increased progesterone exposure in the proximal and distal uterus with the NS compared to Gel (Fig. 3). Furthermore, NS formulation provided > 5-fold increased C_{max} for proximal uterus and > 20-fold increased C_{max} for distal uterus (Supplementary Table 5). Overall, it was notable that the progesterone concentrations in distal uterine tissue were of similar magnitude to proximal uterine tissue after vaginal dosing. Because it is thought that drug reaches the uterus through vaginal absorption, we also attempted to measure progesterone in vaginal tissue that was rinsed of excess product. We found some evidence of increased vaginal tissue absorption with the NS 30 min after dosing (Supplementary Fig. 4), though we did not thoroughly evaluate a full time course due to difficulties ensuring complete removal of residual vaginal product.

3.4. Efficacy of vaginal progesterone in the RU486 mouse model of PTB

It was previously suggested that exogenous progesterone supplementation may not be able to overcome the antagonism by RU486 [36], which is typically dosed on E15 at 150 μg in mice [37]. Thus, we sought to identify whether lower doses of RU486 still caused PTB in the majority of pregnant mice, which may allow for more competition for exogenous progesterone binding. We observed that while the standard dose of 150 μg RU486 and a lower dose of 50 μg RU486 both resulted in 100% PTB on E16, 25 μg RU486 resulted in 85% PTB on E16 (Supplementary Fig. 1). Thus, we used the 25 μg RU486 dose to test the comparative efficacy of vaginal progesterone. Mice were given daily vaginal doses of either progesterone Gel or progesterone NS starting on E15 (black arrowheads, Fig. 4). For RU486, RU486 + Gel and RU486 + NS groups, the median day of parturition was gestation day 16, 16 and 19.5, respectively (Fig. 4). As delivery between E19–20 is considered full-term, the NS group was more efficacious than Gel in preventing preterm birth in the RU486 model. The NS vehicle alone (2% F127) had no effect on PTB, as 100% of mice ($n = 5$) delivered on E16 (data not shown). When comparing the acute prevention of preterm birth on E16 (Fig. 4), 15% of RU486 animals were still pregnant, compared to 47% of RU486 + Gel animals and 80% of the animals in the RU486 + NS group. When we compare the percentage of animals delivering on or after the morning of E19 (full term), 15% of RU486 treated animals delivered at full term, compared to 32% of animals in the RU486 + Gel group and 55% of animals in the RU486 + NS group (Fig. 4). Comparison of the three pregnancy "survival" curves shows a statistically significant difference, log-rank $p = .001$. In multiple pairwise comparisons, only the NS treatment group showed a statistically significant increase in preterm birth prevention compared to the RU486 control group (log rank, $p = .0002$). All animals that delivered at full term gave birth to live pups.

3.5. Effect of vaginal progesterone on cervical remodeling and myometrial contractility

It is known that progesterone plays key roles in term cervical remodeling [38,39]. Having determined that vaginal progesterone dosing was able to prevent PTB in the RU486 model, we then went on to characterize the effects of exogenous vaginal progesterone delivery on the cervix. Oxytocin receptor (*Oxtr*) was previously identified to be upregulated in RU486 model of preterm cervical ripening [40], and we confirmed similar upregulation here using the lower RU486 dose (Fig. 5a). Vaginal treatment with either the progesterone NS or the Gel resulted in a reduction in oxytocin receptor expression in the cervix similar to normal E15.75 mice. Similar increases in gene expression were observed in cervices from RU486 injected mice for steroid 5 alpha reductase type 1 (*Srd5a1*), which is involved in local progesterone metabolism, and connexin 43, a gap junction protein important in the coordination of cell-to-cell communication during transition to cervical ripening (Fig. 5b,c). Further, vaginal treatment with either the progesterone NS or the Gel resulted in a significant reduction in expression of both of these genes (Fig. 5b,c). Interestingly, other genes that were

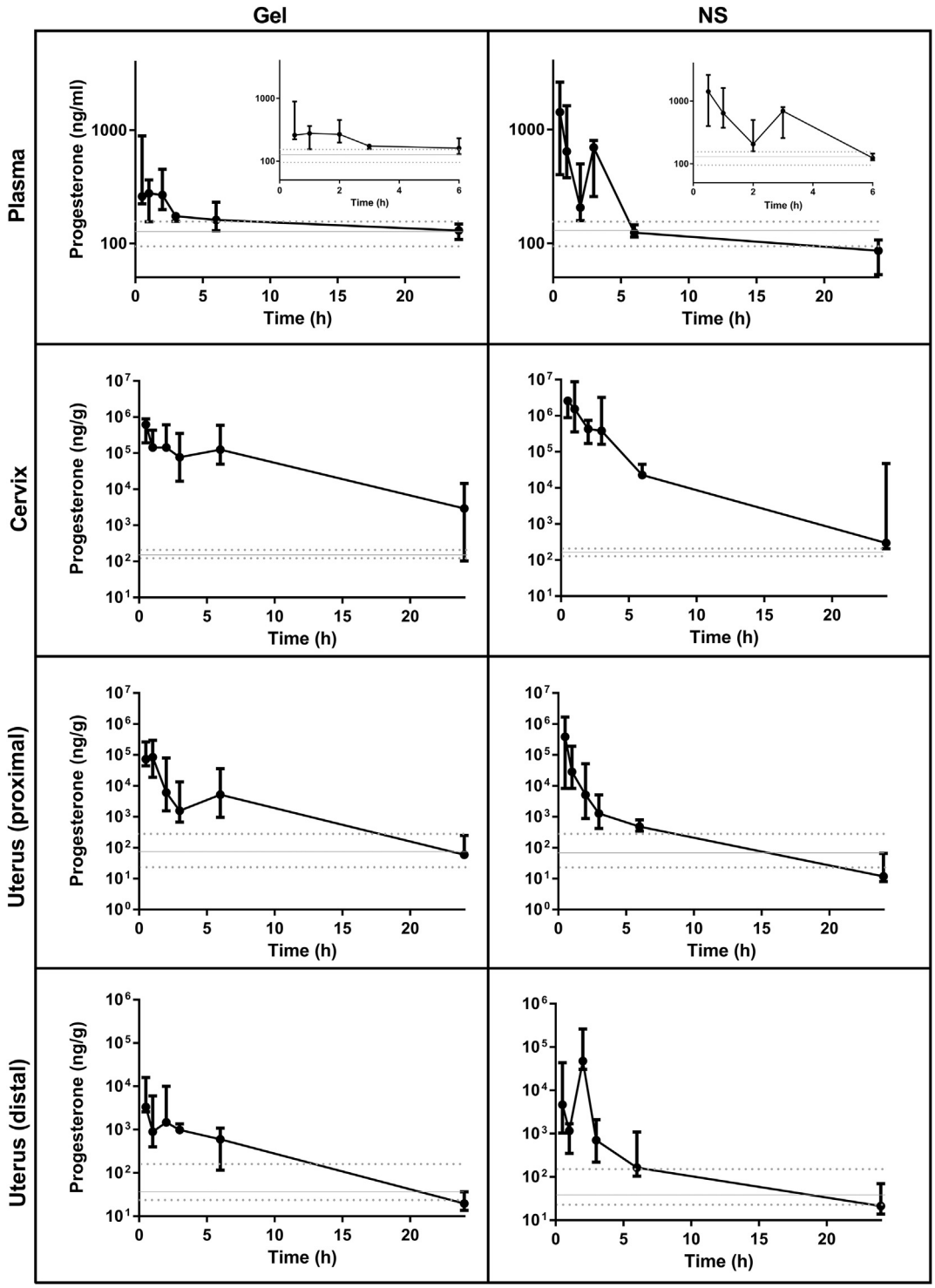


Fig. 2. Progesterone concentrations in various compartments (plasma, cervix, proximal and distal uterus) at selected time points up to 24 h after a single vaginal dose in healthy pregnant mice on gestation day 15 (E15). Insets for plasma show the first 6 h after dosing. Gray solid lines indicate the median progesterone concentrations (± interquartile range; gray dotted lines) measured in samples from healthy pregnant mice on E15 that received no treatment. Data presented as median ± interquartile range for n = 4–5 mice per time point.

previously described to be upregulated in the cervices of pregnant animals dosed with RU486 [40], matrix metalloproteinase 8 (*Mmp8*) expressed by neutrophils and mammalian chitinase (*Chi3l3*) expressed by alternatively activated M2 macrophages, were not found to be significantly upregulated in these studies using a lower RU486 dose (Supplementary Fig. 5). We then looked at another indicator for cervical remodeling, collagen density [41]. Dispersion of collagen fibers is a hallmark of cervical softening, and these morphological changes can be seen with Masson's Trichrome staining (collagen fibers stain blue). RU486 injection led to increased collagen spacing and decreased collagen staining compared to normal E15 cervix tissue (Fig. 6). Treatment with both the NS and Gel resulted in collagen spacing and staining intensity indistinguishable from the normal E15 mice. It is notable that we found little difference in gene expression and collagen remodeling in

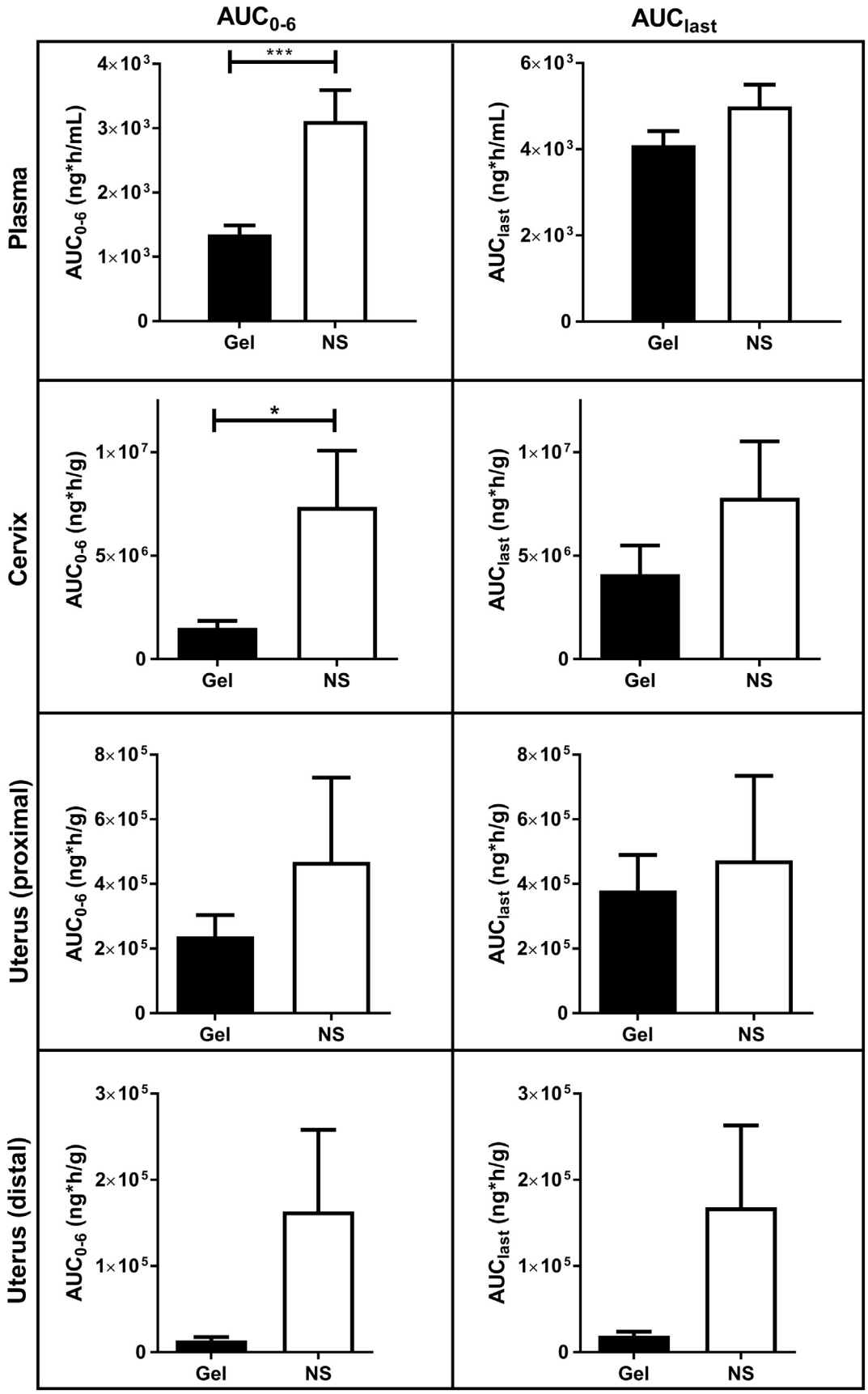
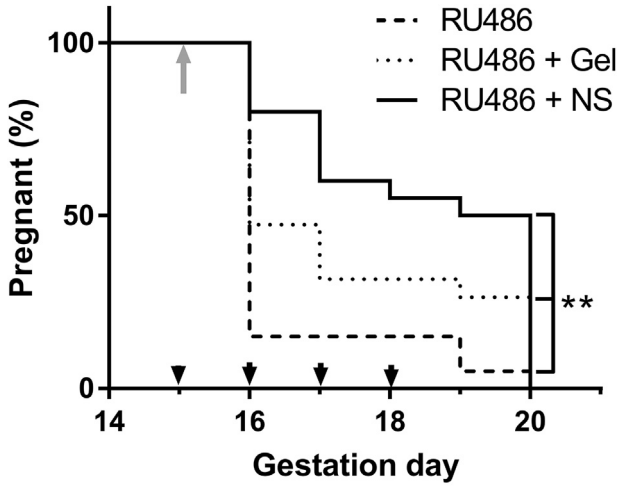


Fig. 3. Area under the curve (AUC) in different compartments for up to 6 h ($AUC_{0–6}$) and over 24 h (AUC_{last}) after a single vaginal dose in healthy pregnant mice on gestation day 15 (E15). Data is presented as average ± SE for n = 4–5 mice per time point. * $p < .05$, ** $p < .01$, *** $p < .001$.



	Median day of parturition	Log-rank p compared to RU486
RU486	16	–
RU486 + Gel	16	0.0528
RU486 + NS	19.5	0.0002

Fig. 4. Percentage of animals remaining pregnant after RU486 injection on gestation day 15 (E15, gray arrow) out of a total gestation of 19.5 days. Animals in the treatment groups received daily vaginal doses from E15–18 (black arrowheads). ** denotes log-rank p = .001 for all curve comparison. Multiple pairwise comparison log-rank *p* value shown below Kaplan-Meier curve. Data shown represents *n* = 19–20 mice per group. – indicates not applicable.

the cervices of mice receiving the NS and Gel treatments, despite the superiority of the NS in the prevention of PTB. It is possible that one key difference could be the increased levels of progesterone reaching the entire uterus after vaginal NS dosing, which could more significantly affect myometrial contractility.

We next characterized gene expression in myometrial tissues. *Oxtr* and connexin 43 are also contractile-associated proteins (CAPs) that are upregulated in the myometrium during both term and RU486 preterm labor [30]. We found that RU486 injection led to upregulation of *Oxtr* (Fig. 7a) and connexin 43 (Fig. 7b) that was partially abrogated by both vaginal progesterone treatments. We also assessed pro-inflammatory cytokines IL-6 and IL-1β and a CAP enzyme involved in prostaglandin synthesis (COX-2) previously described to be upregulated in both term and RU486 preterm labor [30]. However, at the lower RU486 dose used here, we observed no upregulation of IL-6 (Fig. 8a), IL-1β (Fig. 8b) and COX-2 (Fig. 8c) in myometrial tissue at 16 h after injection. However, unexpectedly, myometrium tissue from mice receiving a single vaginal Gel dose had significantly increased expression of IL-6 (Fig. 8a) and COX-2 (Fig. 8c) and a trend for increased IL-1β (Fig. 8b) compared to mice treated with a single vaginal NS dose. This suggests that local toxicity could be a concern for both the vaginal mucosa as well as the uterine environment.

3.6. Effect of vaginal progesterone formulations on the local mucosa

In the context of PTB prevention, vaginal formulations are typically dosed daily. Thus, the potential impact of the formulation on the local vaginal mucosa is important, and causing any unnecessary inflammation or toxicity would be undesirable. Mucicarmine staining revealed that there was an absence of soluble mucus coating the cervices of mice 12 h after receiving one vaginal Gel dose, which was distinct from both RU468 injected mice (preterm remodeling) and normal E19 mice (term remodeling) (Fig. 9). In addition, H&E stained cervices from mice receiving a single Gel treatment had slight attenuation of the epithelium and multifocal apoptotic cells (Fig. 9), which was not observed in any other group. No notable signs of toxicity were observed in H&E stained vaginal tissue sections (data not shown).

4. Discussion

To date, the only clinically proven therapeutic intervention for the prevention of PTB has been progesterone supplementation, with either natural progesterone (vaginal) or 17-hydroxyprogesterone caproate (intramuscular), depending on the target population. In cases of spontaneous preterm birth, sonographic short cervix remains the best predictor of preterm birth risk [42–44]. The PREGNANT trial, a phase III, multicenter, randomized, double masked, placebo-controlled trial demonstrated that daily dosing of Crinone® reduced the rate of PTB before 33 weeks by 44% in women with sonographic short cervix [12]. The subsequent OPPTIMUM trial investigated vaginal progesterone for prevention of PTB in high-risk women (history of PTB, positive fetal fibronectin or cervical length ≤ 25 mm), and concluded that vaginal progesterone did not provide a significant benefit [45]. In response, Romero and coworkers published two systematic reviews and meta-analyses of all randomized controlled trials in women with sonographic short cervix that received either vaginal progesterone or placebo/no treatment, and they concluded that vaginal progesterone provided a significant reduction in preterm birth risk and neonatal morbidity and mortality risk [8,46]. This conclusion is perhaps particularly encouraging considering the various trials used different dosage forms and doses (e.g. 90 mg/day gel application versus 200 mg/day vaginal capsule), suggesting that identifying and universally testing a more optimal vaginal dosage form could provide an even greater benefit.

Here, our goal was to characterize the pharmacokinetics (PK) and pharmacodynamics (PD) of progesterone after vaginal administration

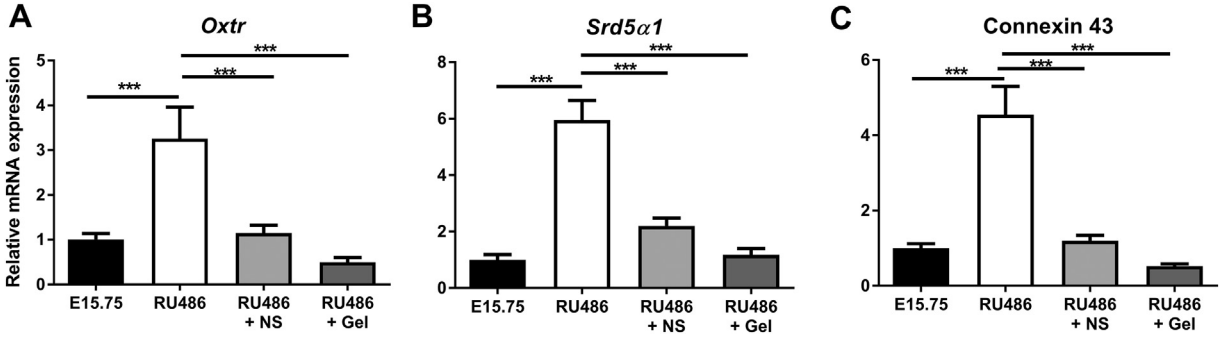


Fig. 5. Expression of genes implicated in cervical remodeling. RU486 injection on E15 results in increased mRNA expression for (A) *Oxtr*, (B) *Srd5α1*, and (C) connexin 43 in cervical tissue compared to non-injected control mice (E15.75). Vaginal progesterone treatment in the form of gel or NS at the time of RU486 injection prevents changes in cervical tissue mRNA expression, resulting in levels similar to E15.75 controls. Data is presented as average ± SEM for *n* = 6–8 mice per group. E15.75 and NS had *n* = 8 per group while RU486 and Gel had 6 and 7 mice, respectively. * $p < .05$, ** $p < .01$, *** $p < .001$.

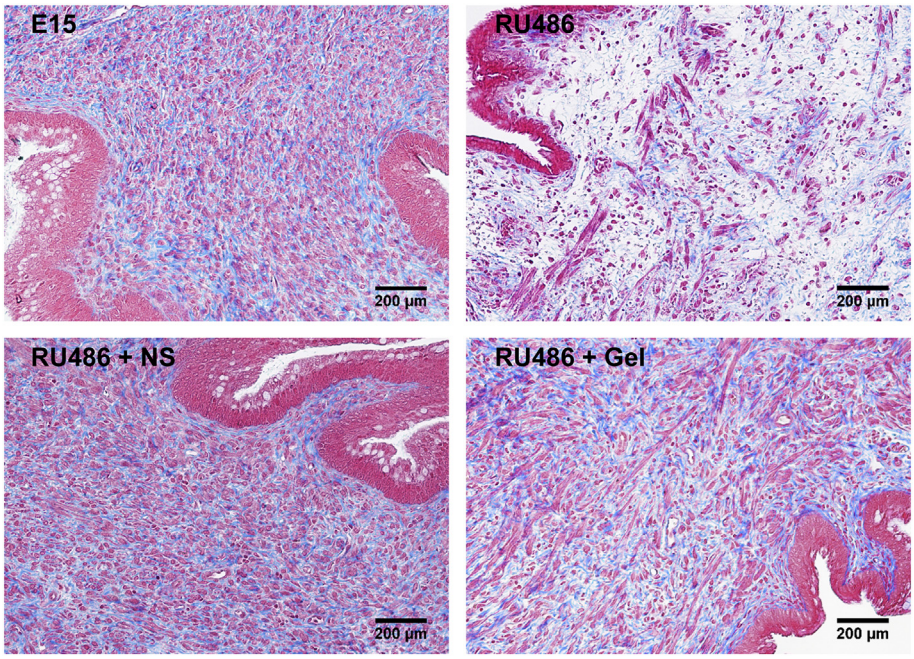


Fig. 6. Cervix tissue sections stained with Masson's trichrome reveal a decrease in collagen density 12 h after RU486 injection, similar to what was previously described. However, vaginal progesterone treatment in the form of gel or NS at the time of RU486 injection prevents dramatic changes in collagen density in cervical tissue, similar to the cervix of a normal pregnant mouse on E15. Images are representative of cervices from $n = 3$ mice in each group.

of Crinone gel and to compare to a formulation designed to provide more uniform vaginal distribution and absorption. Crinone gel is composed of polycarbophil polymer as a "bioadhesive" and contains micronized progesterone (2.5 ± 3.5 μm, Supplementary Table 3) [47]. However, our prior work suggests that particulates that are formulated to be both small enough to fit through the pores in cervicovaginal mucus and coated to be non-adhesive achieve more uniform distribution in the vagina [20]. Further, while micronization of insoluble drugs does lead to increased absorption, nanosuspensions can provide even greater enhancements in water solubility, dissolution, absorption and bioavailability due to the exponential increase in total surface area [48]. Lastly, the excipients in Crinone result in a high osmolality (1587 ± 84 mOsm/kg; Supplementary Table 3) which has been shown to increase local toxicity and osmotically-driven product leakage. In contrast, our formulation was hypotonic, which induces water absorption that distributes mucoinert particulates throughout the vagina and up against the epithelial surface for maximal drug absorption [19]. Our results suggest that the hypotonic, mucoinert progesterone nanosuspension provided improved vaginal progesterone delivery while also being more biocompatible in the vagina compared to the Crinone gel.

There are two proposed mechanisms of action for how progesterone supplementation delays the onset of labor. First, it is thought that the anti-inflammatory properties of progesterone may counteract the inflammatory processes that initiate labor [49]. Second, changes in progesterone receptor expression may lead to a functional progesterone withdrawal that can be offset by progesterone supplementation [50,51]. In mice, circulating progesterone levels decrease to initiate normal labor, while in humans, circulating progesterone levels only decline after birth. It is hypothesized that a "functional withdrawal" of progesterone may occur by a variety of mechanisms, one of the most prominent theories being an increase in the ratio of progesterone receptor A to progesterone receptor B [52]. Nadeem et al. further demonstrated that local/nuclear levels of progesterone were actually low due to increased expression of progesterone metabolizing enzyme 20α HSD [52]. Mifepristone, or RU486, is a competitive progesterone receptor antagonist, which is a model of functional progesterone withdrawal. This model was first described by Garfield and coworkers in rats, where they also observed that twice daily injections of 10 mg of progesterone was not efficacious in preventing preterm birth after 10 mg/kg RU486 injection [53]. Dudley and coworkers later adapted the RU486 model to mice, selecting a dose of 150 μg per mouse to achieve preterm delivery approximately 15–18 h after subcutaneous injection [37]. While various studies characterize the effects of progesterone administration in normal pregnant animals [36,54,55], it was suggested that the higher binding affinity of RU486 for the progesterone receptor rendered the model not useful for testing the effects of progestins on preterm birth [36]. However, the fact that RU486 is a competitive antagonist to progesterone suggests that the dose of RU486 can be adjusted to cause significant preterm birth while allowing the potential to offset its effects with exogenous progesterone delivery. In humans, orally administered mifepristone exhibits nonlinear pharmacokinetics due to saturable protein binding to α_1 acid glycoprotein [56].

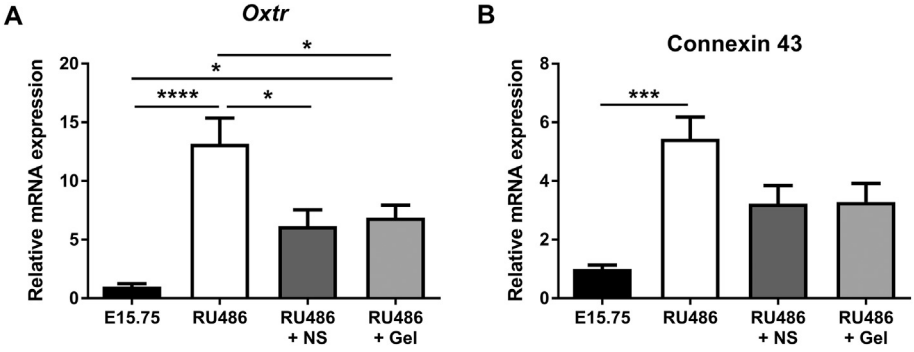


Fig. 7. Relative gene expression of contraction associated genes (CAPs). RU486 injection on E15 results in significantly increased mRNA expression for (A) *Oxtr* and (B) connexin 43 in myometrium tissue compared to non-injected control mice (E15.75). Vaginal progesterone treatment in the form of gel or NS at the time of RU486 injection prevents some of the myometrial activation. Data is presented as average ± SEM for $n = 5$ mice per group. * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$.

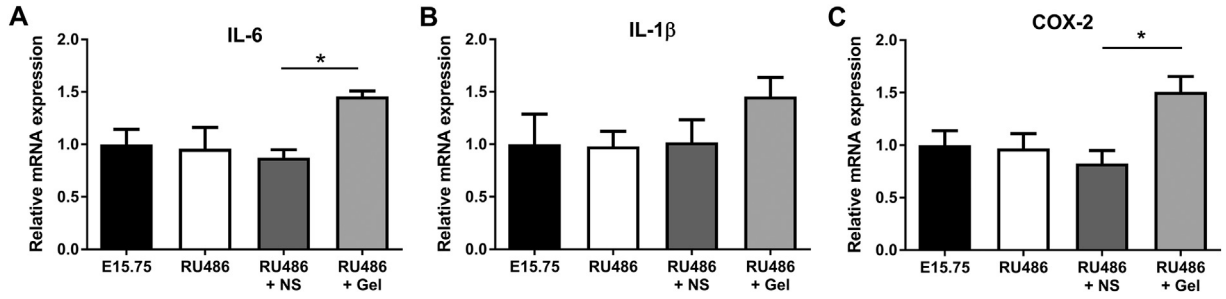


Fig. 8. Relative gene expression of inflammatory cytokines and enzyme regulating prostaglandin synthesis in the myometrium. Increased expression of (A) IL-6 and (B) IL-1β and (C) COX-2 was observed in myometrium for mice receiving a single vaginal Gel dose compared to NS. Data is presented as average ± SEM for n = 5 mice per group. * $p < .05$.

Drug pharmacokinetic nonlinearity may explain why we did not observe an obvious graded response in our dose adjustment experiment (Supplementary Fig. 1). Furthermore, the high RU486 doses may be at the top asymptote of the dose response curve. As we describe here, we observed that a dose of 25 μg RU486 per dam was sufficient to cause preterm birth in 85% of control animals within 24 h, and that the effects of RU486 could be opposed by vaginal administration of 8 mg of progesterone. However, the percentage of dams injected with RU486 that went to full term to deliver live pups was higher in the group receiving the progesterone NS (55%) compared to the Gel (32%), likely because of increased progesterone absorption. These results are in support of the idea suggested in several publications that the optimal progesterone formulation for preventing preterm birth has yet to be identified [9,57,58].

It was previously found that vaginal administration of progesterone led to 10-fold higher concentrations in uterine tissues than systemic administration, despite the fact that the plasma levels were 7-fold lower [11]. For this reason, various vaginal progesterone formulations, including gels, capsules, creams, and tablets have been developed to take advantage of this “uterine first-pass effect” or “first uterine pass effect” [59–61]. It is thought that vaginally administered progesterone is preferentially transported to the uterus before reaching the systemic circulation as a result of countercurrent exchange between the uterovaginal venous plexus or lymphatic vessels and uterine arterties. The fact that we observed relatively high levels of progesterone exposure in the distal (adjacent to the ovary) and proximal (adjacent to the cervix) uterus, despite relatively low systemic exposure, supports the presumption that uterine first-pass also occurred in the pregnant mice. Furthermore, more pronounced double peaks suggestive of recirculation in the distal uterus (2 h) and plasma (3 h) supports that the NS formulation might be more effective than the Gel at taking advantage of the uterine first-pass effect, likely due to increased vaginal drug absorption. A similar double progesterone peak in plasma was observed after vaginal dosing of 8% Crinone gel in healthy postmenopausal women (NDA 20–756, “Fig. 7 90 mg Progesterone Dose”) [62]. Due to limitations in sampling reproductive tract tissues in healthy women, the corresponding uterine tissue concentrations after vaginal dosing of 8% Crinone gel are not available for direct comparison. In pregnant mice, the 1 h delay between the double peak in plasma compared to the uterus suggests that the uterus acts as the “delay” site that provides recirculation [63]. To our knowledge, we are the first to characterize Crinone pharmacokinetics in plasma and reproductive tissues in a preclinical animal model. Our results highlight the difficulty in using blood pharmacokinetics to compare vaginal formulations and predict distribution in the reproductive tract. It is also unclear where progesterone is primarily acting, though it is likely that a number of targets contribute to the therapeutic effect observed clinically [9,38,64–66]. In the preclinical animal model described here, we observed trends of increased progesterone AUC in the plasma, cervix, and proximal/distal uterus with the progesterone NS compared to the Gel. Bailer's method for determining significance with destructive sampling is heavily affected by the inter-animal variability and the pooled estimate of variance [31], which made it difficult to achieve significance for all compartments (Supplementary Table 6). It is also unclear which PK parameter drives efficacy (AUC or C_{max}), although the progesterone NS generally provided higher values for both parameters in all compartments (Supplementary Table 5).

Despite the fact that the vaginal progesterone NS provided improved PK and was more effective in preventing preterm birth compared to the Gel, we found that the NS and Gel had similar effects on the biomarkers of cervical remodeling and uterine contractility that we characterized. It is of note, though, that the markers we chose to evaluate were ones that were previously shown to be elevated in the RU486 mouse model of preterm birth. We did not observe significant increases in *Mmp8* (expressed by neutrophils) or *Chi3l3* (a marker for M2 macrophages) as previously reported at a higher RU486 dose of 500 μg [40]. However, it was demonstrated in mice that the mechanisms driving labor in the RU486 induced preterm birth are different than in normal murine labor, and that there is also a difference in the mechanisms driving preterm birth in the RU486 and inflammation-induced preterm mouse models [40]. Of note, increased *Mmp8* expression was not observed to contribute to normal term cervical ripening [40]. Thus, it is possible that preterm labor is mediated by different mechanisms depending on the dose of RU486, and it is possible that effective progesterone supplementation does not simply reverse or prevent preterm labor processes but acts through other complementary pathways. Furthermore, the temporal dynamics must be key, because RU486 + Gel treated mice had similar expression levels of the genes we characterized in cervical and myometrial tissue compared to the RU486 + NS treatment mice at 16 h, and yet within another 8 h (morning of E16), only 47% of the RU486 + Gel mice had not delivered compared to 80% of the mice in the RU486 + NS group. Migale et al. showed using RNASeq that RU486 mediates two waves of myometrial gene changes at 6 h and 18 h [67]. However, the Migale study used the standard 150 μg dose of RU486, and our results suggest that the dose used for induction would affect the temporal dynamics and the magnitude of changes in gene expression. Another potential factor that could explain why the difference in efficacy was not captured by the gene expression data could be the apparent initiation of inflammatory pathways caused by the Gel formulation.

Regardless of differences in dose and dosage form in different clinical trials, vaginal progesterone dosing typically occurs daily. Thus, the impact of the dosage form on the vaginal mucosa must also be considered. We can look to the vaginal microbicide field for inspiration, as several issues have arisen in the context of requiring generally healthy women at relatively low risk of acquiring an infection to dose a vaginal product daily. First, many vaginal gels and lubricants are formulated with ingredients that result in high osmolality, and the resulting toxicity to the mucosal epithelium have been highlighted as a potential risk factor for increased risk of infection [16,18]. Additionally, discharge and leakage contribute to problems with adherence [68,69]. Although issues with adherence to daily vaginal

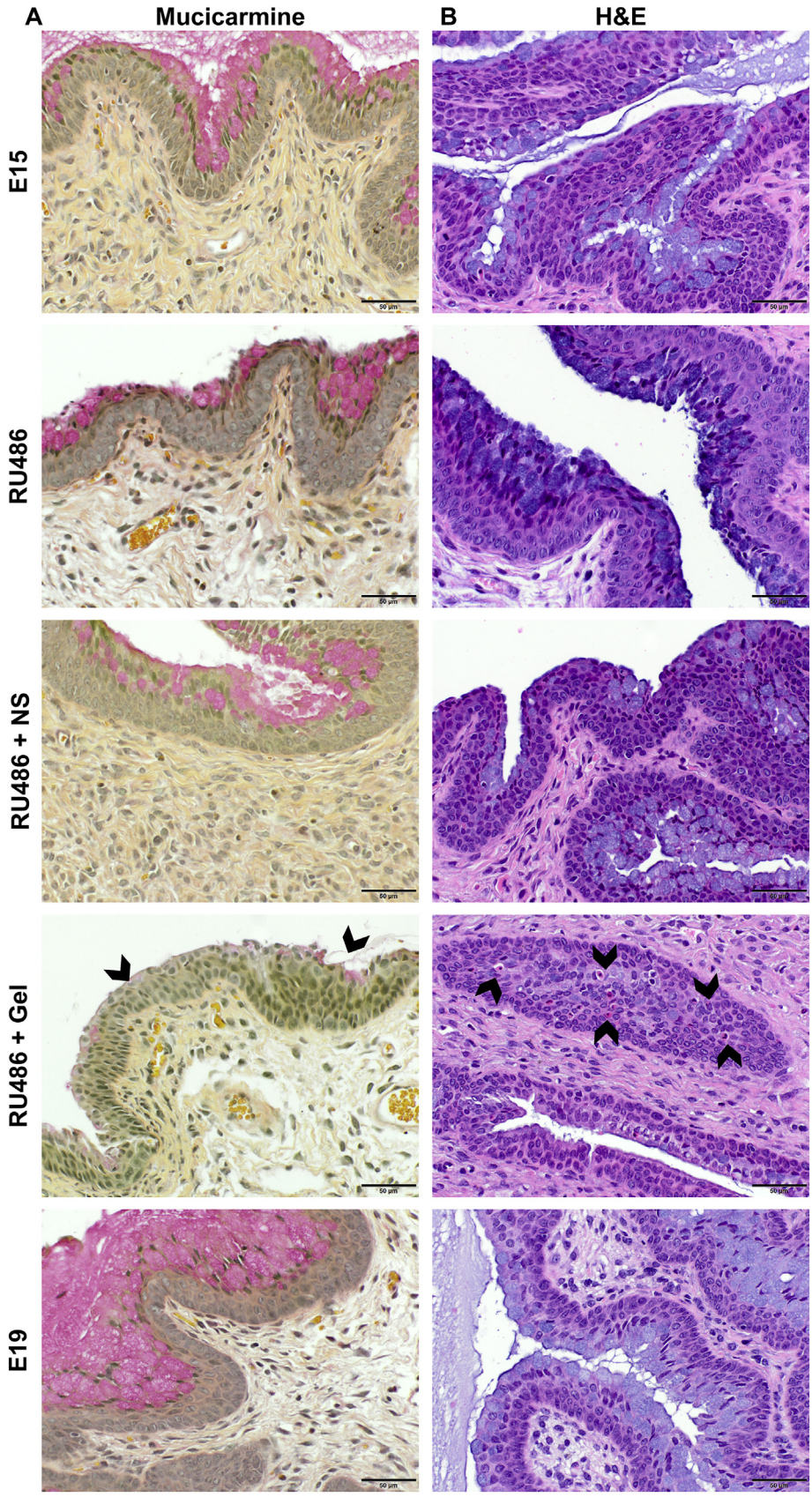


Fig. 9. Cervix tissue sections stained with (A) mucicarmine to visualize mucus and (B) hematoxylin & eosin (H&E) to examine cell/tissue histology. 12 h after vaginal gel dosing, there was a marked decrease in the amount of secreted mucus and evidence of cell apoptosis (marked in images with black arrowheads). The cervical changes observed with vaginal Gel treatment were not evident in normal E15 mice, E15 mice receiving RU486 injection, or normal E19 mice, and were also not observed with the NS treatment. Images are representative of $n = 6$ total mice from 2 replicates (mucicarmine) and n = 3 mice (H&E) per group. Scale bars = 50 μm.

progesterone dosing are not typically cited for women at risk of preterm birth, reducing discharge and leakage can also provide improved drug absorption. Further, it is unclear how local mucosal inflammation may affect the efficacy of a vaginal formulation in preventing preterm birth. We observed cervical cell apoptosis and an apparent reduction in cervical mucus production after one dose of Gel. When characterizing pro-inflammatory genes that were previously shown to be upregulated in the myometrium in term labor and the RU486 model, we unexpectedly observed increases in IL-6, IL-1β and COX-2 only in the group dosed with Gel. It has been reported that IL-1β and IL-6 regulate prostaglandin synthesis through upregulation of COX-2 [70,71]. Further, IL-1β and IL-6 have been implicated in normal and preterm human labor processes [72], and when very elevated, in inflammation-related preterm birth and the associated fetal neuronal injury [73–75]. It is not clear whether signs of local toxicity to the cervix and myometrium led to decreased efficacy in preventing preterm birth in our preclinical animal model, but the potential deleterious effects of inducing a local inflammatory response should be studied further. Overall, our results support the idea that a progesterone formulation that is designed for improved vaginal progesterone absorption while also being more biocompatible could have significant implications in the field of preterm birth prevention.

Author contributions

T.H., M.M. and L.E. contributed to the design of experiments. T.H. and A.D. contributed to the design and development of the NS formulation. S.G. scored the tissue histology slides. N.A. and P.H. developed the analytical LC-MS/MS method and analyzed the samples for progesterone levels. T.H., H.Z., J.O. and L.E. conducted the experiments. T.H. did the noncompartmental pharmacokinetic and statistical analysis. T.H. and L.E. analyzed and interpreted the in vivo/in vitro *data*. H.Z. and N.A. contributed to the methods section. S.G. contributed to the results section. T.H. and L.E. wrote the manuscript. J.H. and J.S. contributed to the interpretation of data and design of follow-on experiments, and provided subject matter expertise and guidance for this project. All authors approve of the final version of the manuscript.

Competing interests

The mucus-penetrating particle technology is licensed and in clinical development for ocular indications by Kala Pharmaceuticals. J.H. is a founder of Kala Pharmaceuticals and serves as a consultant. J.H. and Johns Hopkins own company stock. Under a licensing agreement between Kala Pharmaceuticals and the Johns Hopkins University, L.E., J.H., and the University are entitled to royalty distributions related to the technology. These arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2018.12.046.

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