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Development of a Goat Model for Evaluation of Withaferin A-Cervical Implants for the Treatment of Cervical Intraepithelial Neoplasia

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Abstract

Cervical cancer is caused by human papillomavirus (HPV). The disease develops over many years through a series of precancerous lesions. Cervical cancer can be prevented by HPV-vaccination, screening and treatment of precancer before development of cervical cancer. The treatment of high-grade cervical dysplasia (CIN2+) has traditionally been by cervical conization. Surgical procedures are associated with increased risk of undesirable side effects including bleeding, infection, scarring (stenosis), infertility and complications in later pregnancies. An inexpensive, non-invasive method of delivering therapeutics locally will be favorable to treat precancerous cervical lesions without damaging healthy tissue. The feasibility and safety of a sustained, continuous drug-releasing cervical polymeric implant for use in clinical trials was studied using a

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large animal model. The goat (*Capra hircus*), non-pregnant adult female Boer goats (n=7), was chosen due to similarities in cervical dimensions to the human. Estrus was induced with progesterone CIDR® vaginal implants for 14 days followed by the administration of chorionic gonadotropins 48 hours prior to removal of the progesterone implants to relax the cervix to allow for the placement of the cervical implant. Cervical implants, containing 2% and 4% withaferin A (WFA), with 8 coats of blank polymer, provided sustained release for a long duration and were used for the animal study. The ‘mushroom’-shaped cervical polymeric implant, originally designed for women required redesigning to be accommodated within the goat cervix. The cervical implants were well tolerated by the animals with no obvious evidence of discomfort, systemic or local inflammation or toxicity. In addition, we developed a new method to analyze tissue WFA levels by solvent extractions and LS/MS-MS. WFA was found to be localized to the target and adjacent tissues with 12 – 16 ng WFA/g tissue, with essentially no detectable WFA in distant tissues. This study suggests that the goat is a good large animal model for the future development and evaluation of therapeutic efficacy of continuous local drug delivery by cervical polymeric implants to treat precancerous cervical lesions.

Graphical abstract

Keywords

Cervical dysplasia; cervical cancer; cervical polymeric implant; local drug delivery; goat model

Introduction

Cervical intraepithelial neoplasia caused by human papillomavirus (HPV) infection is characterized by progressive formation of pathologic lesions, known as cervical intraepithelial neoplasia (CIN), or macroscopic lesions like genital warts. The bivalent and quadrivalent HPV vaccines targets HPV type 16 and 18 which causes 70% of cervical cancer. The nonvalent HPV vaccine targets nine HPV types which causes 90% of cervical cancer. The current HPV vaccines are prophylactic and are effective only if administered prior to infection. In 2017, an estimated 12,820 new cervical cancer cases will be diagnosed

and approximately 4,210 patients will die of the disease in the U.S. (Siegel et al., 2017). In the past five years, there has been a substantial increase in early-stage cervical cancer among women under the age of 26. This could be due to early detection and the fact that the extension of coverage that the Affordable-Care Act provides to children up to age 26 to be covered under their parents insurance and therefore receive screening (O'Malley et al., 2006). Although, the incidence of cervical cancer in the U.S. is low the global picture is rather grim. In 2012, it was the fourth leading cause of cancer death with an estimated 265,700 deaths worldwide, of which 90% occurred in developing and underdeveloped countries (ACS, 2015). Clinical data have shown that early diagnosis increases survival since remission rates are less with effective early treatment (Hiom, 2015).

Only women with high-grade cervical dysplasia (CIN2+) are recommended treatment with cervical conization (LEEP). Since genital HPV infection can clear spontaneously, no recommendation is made for specific antiviral therapy. Similarly, treatment is generally not recommended for low-grade CIN lesions, where the standard of care is “watchful waiting”. For high-grade CIN lesions, the only recommended treatment option is loop electrosurgical excision procedure (LEEP). Two to three hundred thousand patients per year undergo LEEP in the U.S., costing a conservative estimate of \$200–300 million annually. Women with cervical precancer treated by conization have a 10–15% risk of recurrence and have to be followed up closely by cervical cytology and HPV-testing (Kocken et al., 2012). Only 12–30% of women with untreated CIN3 will develop invasive cervical cancer during 20–30 years of follow-up (McCredie et al., 2008; van Oortmarssen and Habbema, 1991). This procedure can also result in difficulty in child-bearing (due to decreased fertility) and premature births (cervical incompetence). There is currently no FDA-approved non-surgical treatment for cervical dysplasias. An early medical treatment option that is less invasive and with fewer potential adverse effects, complications and expense than LEEP is needed for patients. Currently, no marketed cervical implant exists for local delivery of pharmaceutically-active compounds, which would prevent the necessity for invasive surgical procedures. Thus, there is an “unmet” need for an intervention therapy which will promise significant reversal of HPV infection as well as cure early- and late-stage intraepithelial lesions.

There are several animal models utilized for the study of cervical pathologies with the majority being murine (Larmour et al., 2015). Testing a localized drug delivery approach for an investigational new drug using rodent models is not translatable to the clinical scenario due to size restraint of the rodent anatomy. This is an important aspect since, for localized drug delivery, the micro-environmental niche dictates the pharmacological action of therapeutic agents. One group reported application of trans-retinoic acid in clinical trial for cervical dysplasia using a collagen sponge in a cervical cap (Weiner et al., 1986). However, this approach only applies for short-term treatment. The lack of appropriate large animal models to evaluate new devices for delivery of compounds at the uterine cervix has hindered the development of new investigational devices. An animal model with a cervix of comparable size to the human cervix was necessary in order to assess the feasibility and safety of sustained-release polymeric implants developed in this laboratory, for the treatment of cervical, vaginal and vulva pathologies. The goat and sheep cervix are comparable to the size and anatomy of the human cervix.

Withaferin A (WFA), a triterpenoid, from the herb *Withania somnifera*, is emerging as a potent therapeutic agent against various cancers, including breast (Stan et al., 2008a; Stan et al., 2008b), prostate (Srinivasan et al., 2007), lung (Aqil et al., 2012), and pancreatic (Yu et al., 2010). Our data showed that WFA down regulated expression of HPV16-associated E6/E7 oncogenes dose-dependently *in vitro* (Munagala et al., 2011). We also demonstrated antitumor activity of WFA against cervical cancer xenograft in nude mouse model. Further, analysis of tumor tissues lysate for HPV oncogenes E6/E7 and tumor suppressor genes p53/pRb showed downregulation of oncogenes with associated upregulation of tumor suppressor genes, respectively (Munagala et al., 2011).

The objective of this study was to optimize polymeric implants (“cervical inserts”) for continuous (“24/7”) release of WFA at the target site (i.e., the cervix) using the goat as the animal model. The studies described represent the necessary pre-clinical efforts to examine potential toxicity and rate of release of WFA from the cervical implants and assess tissue distribution. In addition, the concept of local continuous delivery of therapeutics is a novel and innovative concept that has the potential to revolutionize the clinical management of high-risk individuals.

Materials and Methods

Chemicals

ε-Polycaprolactone of average mol. wt. 80,000 (P-80) and polyethylene glycol of mol. wt. 8,000 (PEG-8,000) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pluronic® F68 (F68) was a gift from BASF Corp (New Jersey, USA). WFA was isolated from the Ashwagandha extract (Sabinsa Corp, NJ, USA) in the laboratory and was purified by solvent extraction, followed by C18 and Sephadex column chromatography to over 96% purity, as confirmed by HPLC. All other chemicals used were of analytical grade.

Formulation of cervical implants

The polymeric materials, with and without WFA, were prepared and cylindrical implants extruded using appropriate molds as described earlier (Aqil et al., 2012; Gupta et al., 2012). Cervical polymeric implants were prepared as follows: Sham cervical inserts were prepared by dissolving P-80:F68 (9:1) in dichloromethane, which was removed by the evaporation in a Petri dish inside a fume hood, followed by speed-vac (Thermo-Savant, Holbrook, NY) under high vacuum. The polymeric material was then filled in a disposable syringe and the assembly was heated for 15–20 min at 70 °C. The molten material was injected into specially-designed molds and implants were removed after cooling. WFA-loaded implants were prepared similarly by dissolving the desired concentration of WFA in dichloromethane and mixing with the polymer solution prior to drying.

The WFA implants were coated with 6–10 layers of 10% P-80 solution in dichloromethane to circumvent early burst release as described (Aqil et al., 2012). Coatings were added by dipping the implant into the blank polymer solution with intermittent drying with cool air using a commercial hair dryer and placing them inside of a fume hood. The implants were

left overnight to remove the residual dichloromethane and stored in amber vials under argon at -20°C until used.

***In vitro* drug release**

The rate of WFA release *in vitro* for cylindrical implants was measured as described (Aqil et al., 2012). To determine the rate of release of WFA from cervical inserts, the implants about 1.9 g in weight ($n = 3$) were placed in 100 ml PBS containing 10% bovine calf serum and 1% penicillin-streptomycin solution in 250-ml amber glass bottles. The bottles were placed in a water bath (Julabo SW 23, Seelback, Germany) at 37°C with constant agitation at 110 rpm. The medium was changed every 24 hour. WFA was extracted from the medium with acetonitrile and chloroform. Briefly, to 1 ml of release media was added 2 mL acetonitrile. Precipitated proteins were separated by centrifuging at $10,000 \times g$ for 10 min, supernatant was extracted with two $\times 4$ ml of dichloromethane. The organic phase was pooled and dried in speed-vac overnight. The samples were reconstituted in acetonitrile and 5 μL samples were analyzed on UPLC system as described (Munagala et al., 2016).

Ultra performance liquid chromatography

We used a gradient of acetonitrile and water on a Shimadzu Shim-Pack XR-ODS II reverse-phase column (150×3.0 mm i.d., $2.2 \mu\text{m}$) at a flow rate of 0.7 ml/min in which acetonitrile concentration was increased from 5% to 60% from 1.3 to 5.1 min, followed by an increase to 80% from 5.1 to 7.7 min and finally to 100% at 10 min. The latter ratio was then maintained till 10.9 min and finally decreased to 5% in 12 min. The WFA was detected by photodiode array (PDA) detector at 215 nm and the total concentration was calculated against the standard curve of WFA. The detection limit was found to be 0.3 ng.

Animal experiments

This study was conducted in accordance with applicable institutional and national guidelines and regulations for the care and use of animals in research. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville prior to initiation. The UofL animal care and use program is fully accredited by AAALAC, International.

Adult female Boer goats ($n=7$) were procured from pH Farm (Minster, OH, USA). All goats were non-pregnant and Q-fever (*Coxiella burnetii*) negative as confirmed by pregnancy-specific protein B (PSPB) serum assay and ELISA screening, respectively. Animals were group-housed on solid floors with hard-wood shavings at a temperature of $68\text{--}72^{\circ}\text{F}$, a humidity of 30–50%, 100% fresh air changes were conducted within the housing room at a rate of 10–15 per hour, and a 12:12 light cycle. All animals were acclimated to the facility and husbandry for a minimum of two weeks prior to experimental manipulation. Animals were fed with Rumilab[®] 5508 (Purina LabDiet[®], Cincinnati, OH) at 2% of the animal's body weight divided into two daily meals (adjusted as needed to maintain body weight). The animals were also provided timothy hay and water *ad libitum*. Environmental enrichment included plastic and rubber balls, inner tubes suspended from chains, and rubber kong-type toys on the ground and suspended from chains.

Two animals served as untreated controls for histopathology, CBC, and serum biochemistries that did not receive cervical implants. Five animals served as their own sham implant controls (received cervical implant without WFA), then received no treatment for one month, followed by implantation of WFA loaded cervical implants (n=3 received 2% WFA-loaded implants and n=2 received 4% WFA-loaded implants).

Induction of estrus to relax cervix for implant placement

Goats are seasonally polyestrous and outside of the natural breeding season, therefore adjunctive methods must be utilized to induce estrus to relax the cervix sufficiently to allow placement of a cervical implant. Common hormone treatments consist of the administration of a progestin transvaginal implant or pessary for 7–14 days followed by human chorionic gonadotropin (hCG) and equine chorionic gonadotropin (eCG) for 48 hours prior to removal of the progestin (Mobini S et al., 2002).

Briefly, a 0.3 g progesterone controlled transvaginal drug releasing device (Eazi-Breed™ CIDR® for Sheep and Goats, Zoetis, Florham Park, NJ) was placed intravaginally in three animals following manufacturer's instructions for 7 days. The perineal area of the animals was cleaned gently with a dilute (1:40) chlorhexidine solution prior to placement of the CIDR. Forty-eight hours prior to removal of the CIDR® each animal received an intramuscular injection of 400 IU equine chorionic gonadotropin (eCG) and 200 IU human chorionic gonadotropin (hCG), (PG600®, Intervet, Millsboro, NC). However, we observed that the cervix was not dilated sufficiently for the placement of the device. Following this, in five animals, including the three animals where estrus was not sufficiently induced for cervical implant placement previously, the same protocol was used as described above, but the progesterone CIDR® was left in place for 14 days. Both groups of animals were monitored daily post CIDR® placement for attitude, mentation, appetite, fecal and urine output, respiration rate, vocalization, vaginal/vulvar discharge or bleeding, and for the presence of the tag at the end of the CIDR® to insure its continued placement, until the transvaginal implants were removed. This procedure was sufficient to consistently relax the cervix for the placement of the cervical implants.

Cervical biopsy and implant placement

All instruments and supplies were either steam or gas sterilized prior to use. Two days following CIDR® removal, following an overnight feed and water fast, the animals were sedated with 0.03 mg/kg xylazine SC. Intravenous catheters were placed in a marginal ear vein and animals were anesthetized with 5.0 mg/kg ketamine and 0.4 mg/kg midazolam IV and anesthesia was maintained with 2.0–3.0% isoflurane through an endotracheal tube (7.0 mm). Blood pressure was maintained throughout the anesthetic procedure with lactated ringer's solution (5–10 ml/kg/hr, IV) through an IV catheter placed in a marginal ear vein. Buprenorphine (0.005 mg/kg, IV), carprofen (2.0 mg/kg, IV) and cefazolin (20 mg/kg, IV) were administered prior to initiating the cervical procedure. Body temperature was maintained with warm air circulating blankets. Non-invasive blood pressure, body temperature, SpO2, end tidal CO2, HR and RR were monitored continuously throughout the anesthetic procedure.

Blood samples were collected for baseline CBC and serum chemistry from the jugular vein. The tails of the animals were retracted cranially (hemostats clipped to the hair of the animal's tail and back), to provide clear access to the perineal area. Hair in the perineal area was clipped, and the skin of the vulva and perineum was aseptically prepared with chlorhexidine surgical scrub, alcohol, and chlorhexidine solution. A sterile surgical drape was placed over the perineum of the animal so that only the vulva was exposed.

Schroeder tenaculum forceps (10", straight) were placed on the cervix for stabilization and manipulation. Cervical biopsies were obtained from each animal with Tischler oval biopsy forceps at the 12 O'clock position of the cervix prior to cervical implant placement. Hemostasis following biopsy was achieved with extra-large 6" cotton tipped applicators. On rare occasion styptic powder was additionally necessary. Hegar cervical dilators (3 mm–12 mm) lubricated with a sterile water-based lubricant were used to dilate the cervix to a diameter that would accommodate the stem of the cervical implant beginning with 3 mm and gradually increasing in size.

Five of the cervical implants were sutured to the cervix and/or surrounding vagina with 2–0 Nylon suture material with simple interrupted sutures. The hooves of the animals were checked following the procedure and were trimmed as necessary. The animals were then recovered from anesthesia. Carprofen (2.0 mg/kg, s.c.) was continued once daily for 48 hours post procedure for analgesia. Animals were monitored daily for attitude, mentation, appetite, respiration rate, vocalization, and vaginal/vulva discharge or bleeding. Sham cervical implants were placed for one week in three animals, and for two months in two animals. Following a one month resting period, three animals received 2% WFA implants and two animals received 4% WFA implants placed for 3 months. Cervical biopsies were performed immediately prior to placement of the sham implants. WFA-implanted animals also received biopsies immediately prior to and one month post implant placement. Biopsy locations were alternated between the 12 O'clock and 6 O'clock position of the cervix. At the end of the study, animals were humanely euthanized and tissues were collected for histopathology and drug distribution.

Measurement of residual drug (*In vivo*)

Post treatment, implants were recovered from the goats and dried overnight under vacuum. The residual contents of the WFA in the implants ($n = 3$) were analyzed by dissolving the implant in 100 mL dichloromethane, and diluting with ethanol (1:1). The polymer pellet was discarded following centrifugation at $12,000 \times g$ and the supernatants were diluted with ethanol:dichloromethane (80:20). The WFA concentration was measured by UPLC and the concentration was calculated against the standard curve of WFA, as described for *in vitro* release.

Analysis of tissue WFA

Methods were developed for the extraction of the tissue WFA. Briefly, 500 mg each of local, adjacent (cervix, vagina, vulva) and other distal tissues collected from control animals were spiked with known quantities of WFA. Tissues were homogenized in 2 ml PBS (pH 7.4) and 2 volume (4 ml) of acetonitrile was added to precipitate the proteins. The supernatant was

extracted with 2 volumes (8 ml) of chloroform and the organic phase was dried in speed-vac. The dried residue was dissolved in 30 μ l of methanol, filtered through 0.2 μ m spin filter and 15 μ l was injected in UPLC for analysis. The method was highly reproducible with minimum inter- and intra-day variability and provided much higher (73%) extraction efficacy. Using this method, we extracted WFA from goat tissues and analyzed by UPLC as described above. However, the levels in experimental tissues were below the detection limit of UPLC.

LC/MS-MS analysis

To achieve a reliable low level detection, a new method was developed to analyze WFA in animal tissues by LC/MS-MS. WFA was dissolved in 95% methanol: 5% 2 mM ammonium acetate in water and the internal standard, prednisolone (Sigma-Aldrich, St. Louis, MO) was first dissolved in 100% ethanol, and then both the solutions were diluted to 40 μ M in the 95:5 methanolic solution. The compounds were then infused into a Waters (Milford, MA) Quattro Premier XE triple quadrupole mass spectrometer and optimized ionization conditions were determined as well as optimal daughter ions used to program sensitive Multiple Reaction Monitoring (MRMs). The voltage applied to the source capillary was 0.8 kV and the source cone voltage was set at 25 V. The extractor voltage was 3 V and the RF lens was 0.0 V. The source and desolvation temperatures were 120 °C and 500 °C, respectively. These settings along with a nitrogen desolvation gas flow of 900 L/hour produced an optimized number of ions of each compound to reach the mass filters. The MRM transitions used for WFA were m/z 471->95.1 (quantification transition), 471->281.2 (confirmation transition), and 471->299.3 (confirmation transition). For prednisolone the MRM transitions used were m/z 403->147.2 (quantification transition), 403->307.3 (confirmation transition) and 403->385.3 (confirmation transition).

Next these solutions were injected onto an Acquity HSS T3 (2.1 $\times$ 50 mm 1.8 μ m particle size) column using a Waters Acquity UPLC. The compounds were eluted from the column kept at 40 °C and into the mass spectrometer using a binary solvent system consisting of 2 mM ammonium acetate for A and 100% methanol for B. The gradient was as follows: Initial conditions were 90:10 A:B ramping to 5:95 A:B over 3 min then the solvents were stepped back to initial conditions and the column was equilibrated for 2 minutes. The flow rate of the solvents was 0.55 mL/min.

Toxicity studies

After placement of the implants, animals with implants longer than 1 week were anesthetized every 30–60 days to check the placement of the cervical implants, and collect blood for CBC and serum chemistry. Blood was also collected separately just prior to necropsy, and hematological parameters were analyzed using whole blood by Cell Dyn 3500 hematology analyzer (Abbott laboratories, Santa Clara, CA). Serum was used to analyze the liver and kidney function test as described elsewhere (Munagala et al., 2016).

Statistical analysis

All the statistical analysis was carried out using Graph-Pad Prism (La Jolla, CA, USA) and Microsoft Excel 2013. The data were further analyzed for statistical significance using student's t-test. A p-value of < 0.05 was considered significant.

Results and Discussion

The purpose of this study was to evaluate the goat as a potential large animal model for the preclinical evaluation of cervical implants for local delivery of therapeutics. Adult female Boer goats (*Capra hircus*) were used to evaluate feasibility, biocompatibility, and toxicity of cervical implants (both sham and WFA implants) placed within the cervix as a potential treatment for human cervical cancer.

Induction of estrus to relax cervix

The Boer goat was chosen for the similarity in cervical dimensions to the human cervix (Lyngset, 1968). Since the cervix of the goat is tightly closed when the animals are not in estrus, precluding the ability to place the cervical implant, it was necessary to induce estrus (Dayan et al., 2010). Several methods have been described in the literature, which include manipulation of the length of daylight exposure, administration of melatonin, exposure to a ram, and hormone administration to induce and synchronize estrus, which is commonly used when performing artificial insemination (Mobini S et al., 2002). We chose hormone administration for its high reproducibility. The method involves a progesterone intravaginal drug-eluting insert (CIDR, controlled intravaginal drug release) in conjunction with treatment with a combination of equine and human chorionic gonadotropin to elicit estrus and relaxation of the cervix to allow placement of the cervical implant. The standard protocol for artificial insemination of seven days of progesterone CIDR® treatment was insufficient to relax the cervix for placement of the cervical implant. We found that in order to consistently induce estrus that resulted in a cervix that is relaxed enough to accept placement of a cervical implant, 14 days of exposure to a progesterone vaginal implant followed by chorionic gonadotropins was required. Modifications of the original prototype cervical implant design were necessary to accommodate the caprine cervix differences from the human cervix, including a small pelvic canal limiting access to the cervix and manipulations, fibrocartilaginous rings, tight closure of the cervix in the absence of estrus.

Development of cervical implantable device

Before cervical implants loaded with WFA were tested, we used sham implants first to identify the most appropriate implant design. Figure 1 depicts the various implant designs that were fabricated and tested. Suitability of the implant or lack of it was based on the following criteria: i) the ease with which the implant could be inserted into the cervix; ii) whether the implant was retained in the cervix iii) toleration of the implant by the goats based on food consumption, the presence of straining, tail flagging, or vaginal discharge, mentation, fecal and urine output, decreased activity and weight loss. It required at least 5 different implant designs before finding the optimal design: The original prototype implant with a cylindrical shaft (Fig A–C, D; Design 1–2) was developed for human cervix. Since the implant was too smooth it did not remain in place within the goat cervix. As previously

discussed, unlike the human cervix the goat cervix has additional fibromuscular rings and folds that hold the cervix very tightly closed when the animal is not in estrus (Dayan et al., 2010). Initially, we presumed these rings would tighten upon the implant to hold it into place as does the human cervix, however, we found that goats push the implant out of the cervix. Therefore, different implant designs with ridges and/or bulges were fabricated (Fig 1D; Design 3–5) to improve implant retention within the cervix, however none satisfied all the criteria listed above. The implant design 3 cap was also made rectangular on two sides so a long device could assist with implant placement. We then fabricated a metal ejector (Fig 1E) which was inserted into a small cavity in the cap of the implant (Fig 1D; Design 4–5). This assembly helped greatly in inserting the implant into the cervix. The implant design 5 (Fig 1D; Design 5) was found to be most appropriate in that in two of the three goats the implant remained in the cervix but in one goat the implant was found to slide out after about three weeks. We finally drilled two holes in the cap of the implant and after its insertion into the cervix, two stitches were added using non-absorbable suture (1–0 Ethilon), one on each side of the implant cap. We believe that the cervical implants were well tolerated by the animals with the exception of the sham implant with the threaded stem (Fig 1D; Design 4). This design appeared to cause the animal discomfort, inflammation, and potentiated a bacterial vaginitis/cervicitis and was discontinued. The animal responded well to analgesic therapy. All other animals appeared to be comfortable following placement of the cervical implant. The implant that worked best was the second redesign with the bulbed stem (Fig 1D; Design 5). All animals had a good appetite, mentation, activity level, and maintained or gained weight during the study. In addition to the animal with the threaded stemmed implant, one animal with a 2% WFA implant developed a vaginitis. A bacterial culture and sensitivity was performed on both animals, followed by treatment for two weeks with an appropriate antibiotic based on the results. At a one month evaluation period post treatment initiation, the vaginitis was resolved in both animals. We suspect the vaginitis in the 2% WFA implant developed secondary to inadvertent introduction of bacteria during the implant procedure, and not due the presence of the implant itself.

Coated implants and *in vitro* release

We had previously shown that polymeric cylindrical implants designed for rodent studies were accompanied with an initial burst release (Aqil et al., 2012; Gupta et al., 2012). We tested if the burst release phenomenon could be curtailed by adding multiple coatings of blank polymer. We used 6 and 10 coatings of blank polymer on the WFA cylindrical implants and determined the rate of *in vitro* drug release. The release was conducted in 10 ml media. The *in vitro* release was done in PBS containing 10% bovine serum in the release medium. The WFA was found to be released with a burst effect initially in the absence of any blank coats; however, the burst effect phenomenon was substantially arrested by the blank polymer coatings and the degree of the arrest was proportional to the number of the coatings. Six to 8 blank coats were considered optimal as higher numbers, in fact, lowered the initial rate of release (Fig. 2).

Based on these findings, cervical implants were prepared with 2% and 4% of WFA load and added 8 blank polymer coatings. Since the size of the implants (~ 2 g wt.) was almost 5 times of cylindrical implants, the release was conducted in 100 ml media. The burst release

from the coated implants on day one was found to be reduced to about one half ($397 \pm 41 \mu\text{g}$ WFA) compared with the implants with no coatings ($686 \pm 176 \mu\text{g}$ WFA). However, the amounts released from the two types of the implants were essentially similar after five days. In the span of 30 days, implants released a total of 11.8% and 7.7% WFA in the absence and presence of the blank polymer coatings, respectively (Fig. 3).

Cervical Biopsies

Blood and cervix biopsy samples at time zero (before placing the polymeric device), 1 month and at the time of euthanasia, collected from untreated goats and goats treated with sham, 2% and 4% WFA implants, were analyzed for any systemic and tissue toxicity. The target (cervix), surrounding (vulva and vagina) and distal (ovary, kidney, lung, liver, etc.) tissues collected at 3 months at euthanasia were also examined histologically.

Cervical biopsies were evaluated by a board certified veterinary pathologist. Each tissue specimen was cut into 2 pieces and both pieces were examined in their entirety microscopically and representative images with higher drug load are shown in Fig. 4. All samples were examined after blinding the samples. Cervical biopsies from sham and 2% WFA implants collected one month after implant placement and at the time of necropsy showed no evidence that would suggest a toxic or foreign body reaction, systemic inflammation, ulceration or necrosis. The biopsies collected from one goat that received a 4% WFA implant at 30 day time point did not show any adverse reactions. However, a biopsy at 90 days showed mild edema in one of three goats but no significant localized toxic reaction was observed. There were no abnormalities on cervical histopathology of the other animals at the time of necropsy. There were some signs of mild hemorrhage in the cervical biopsies which presumably occurred at the time of biopsy; inflammation was also mild.

Histopathology revealed no evidence of significant inflammation or cellular necrosis suggesting no evidence of local toxicity. The only abnormal findings during gross necropsy were in the two 4% WFA implanted animals which consisted of a large amount of clear mucus surrounding the cervix and within the vagina. These findings suggest the sham and 2% WFA implants were well tolerated by the goats as none of the animals exhibited any symptoms associated with discomfort or abnormal vaginal discharge associated with the presence of the implant, and all groups of animals were clinically healthy throughout the study. In addition, all animals had normal feed intake throughout the study and maintained or gained weight based on weekly measurements.

Serum chemistry and complete blood count

Animals were observed for any possible systemic toxicity due to the polymeric materials (sham implants) or WFA (WFA implants). All CBCs were found to be normal in the WFA-treated goats compared with sham treatments throughout the study, as well as in the sham implant-treated goats compared with untreated control goats, indicating lack of inflammation associated with the implant materials or WFA itself (Fig 5). No significant differences in various hematological parameters (white blood cells, red blood cells, hemoglobin), liver enzymes except alkaline phosphate (like aspartate transaminase, alanine aminotransferase, gamma glutamyl transpeptidase, amylase, or lipase), or biochemical

parameters of kidney function (like blood urea nitrogen, creatinine, Ca^{2+} , Na^+ , and Cl^- levels) were observed with WFA treatment after 1, 2 and 3 months of grafting (Fig 5). The alkaline phosphate was elevated in three of the five animals, however, the change was statistically insignificant. Since these animals were not clinically ill, and there were no additional liver enzyme elevations, it is suspected that the CIDR® and the stage of the reproductive cycle may be the reason for the elevation (Chang-You YU et al., 2011), however further investigation in future studies into the elevation with assessment of origin specific isoenzymes may be warranted. Furthermore, neutrophils, lymphocytes, monocytes, eosinophils, and basophils were also not affected by the WFA treatment or the device. These results clearly indicate a lack of any systemic toxicity or foreign body reaction due to the polymeric implant or WFA.

***In vivo* drug release**

Implants were recovered from the animals and analyzed for the amount of the residual WFA. As expected, no physical changes were observed in the implants at the end of 3 months. The 2% WFA implants were found to release a total of 12.7 – 12.8 mg over the period of 3 months out of 38 mg WFA embedded in the implant of about 1.9 g (Fig 3). Thus, the average daily release was $142 \pm 5.6 \mu\text{g}$; the 4% WFA implants gave a total release of $28.7 \pm 1.6 \text{ mg}$ over 3 months out of 76 mg WFA embedded in the implant weighing 1.9 g, thus an average daily release of approximately 312 ± 17.5 (Fig 3). These data indicate that only one third of the WFA present in original implants was released over 3 months, implying the WFA implants could continue to provide the drug release for much longer duration.

Tissue bioavailability

Due to lack of published methodology to detect WFA in tissues, systematic efforts were made to develop a methods to measure WFA in tissues:

When we adapted the reported HPLC method (Agrawal et al., 2015; Patial and Gota, 2011; Patil et al., 2013) to UPLC using PDA-UV detector patterned, we reached a detection limit for WFA at 0.3 ng. This detection limit was substantially lower than the detection limit of 34 ng reported (Agrawal et al., 2015). This improved detection limit is presumably due to the use of a column with 2.2 micron particle size and a high pressure system. This methodology was sufficient to measure the *in vitro* release and residual WFA in the implants.

However, we were unable to detect WFA in the tissues using HPLC. To achieve a reliable ultralow-level detection required a major alteration in the methodology, i.e., by LC/MS-MS. Figure 6 show representative mass spectra of WFA and its daughter ions. We included prednisolone as an internal standard since deuterium-labeled WFA was unavailable at that time. The method showed excellent linearity ($R^2 > 0.99$) and the lower limit of quantification was found to be 0.02 pg. The validated method was successfully applied to the tissue distribution study.

We developed methods for extraction of WFA from tissues focusing on suitable solvents, extraction efficiency, and reproducibility. The method was highly reproducible with minimum inter- and intra-day variability and provided acceptable (73%) extraction efficacy. The tissue distribution showed as expected higher levels of WFA at the site of implantation

and in adjacent tissues (Fig. 7, Supplementary Fig S1–S5). The tissue levels of WFA were found to be dose dependent with 10–16 ng/g tissue from 4% WFA implant and 4–7 ng/g tissue from 2% WFA implant in the cervix and adjacent tissues. The distal tissues (liver, lung, ovary, kidney, brain and spleen) as well as blood showed no detectable WFA except for the lung which showed traces of WFA in one of the three goats with the higher dose (4% WFA implants). These data indicate the cervical implants delivered the test agent (WFA) essentially locally, with little or no systemic delivery.

Despite several studies reported on the therapeutic efficacy of WFA against various cancers, including cervical cancer, no analytical methods have been reported to the best of our knowledge to measure tissue levels of WFA. Blood WFA levels have been reported in early time points (up to 24 hour) following a bolus dose (4 mg/kg) of WFA in rodents and showed that WFA was undetected beyond 6 hours (Thaiparambil et al., 2011).

Cervical cancer chemotherapy typically costs \$10,000 to \$200,000, depending on adjuvant drugs used, the mode of delivery and the number of treatments required. A study published in the J. Gynecologic Oncology reports that a commonly used treatment for cervical cancer is cisplatin combined with radiation which typically costs about \$40,000, while adding the drug gemcitabine increased the cost to over \$60,000. In contrast, the drug-loaded cervical implant device is estimated to cost only \$500 – \$1,000 (Phippen et al., 2012). Cisplatin is one of the most effective chemotherapeutics against many forms of cancer and for cervical cancer this is the first-line chemotherapy. Despite its ubiquitous use cisplatin is associated with significant dose-limiting toxicities including nephrotoxicity, ototoxicity and neurotoxicity. These severe side effects are attributed to higher required doses due to the vulnerability of cisplatin to complex with plasma proteins leading to deactivation, and reduced therapeutic efficacy. There is a clear incentive to develop new strategies for safer and more effective chemotherapy. This proposed local delivery approach is considered a “game changer” for the treatment of cervical cancer; it applies for the treatment of vaginal and vulva cancers as well, since the vulvar and vaginal tissues showed high levels of WFA similar to that found in the cervical tissue (Fig. 7). Though the implant delivery was devised for loading WFA for the treatment of high-grade cervical dysplasia (CIN2+), the implant can be loaded with potentially any therapeutic drugs that are commonly used for the treatment of cervical, vaginal and vulvar pathologies.

Our polymeric implant technology for local delivery will allow cost-effective treatment of cervical intraepithelial neoplasia including cervical cancer with little or no off-target side effects. The approach to deliver drugs directly at the vaginal or cervical site for higher efficacy and bioavailability has been reported before. Ruffin and colleagues reported utilizing female condom or cervical cap to deliver all-trans retinoic acid absorbed in collagen sponge (Ruffin et al., 2004). The cervical cap was replaced daily for up to 5 days by trained nurse practitioner. In a clinical trial that concluded in November 2013 (NCT01035580), a group in Emory University studied the safety and pharmacokinetics of intravaginal curcumin delivered daily for 14 days. They used curcumin capsules containing 500 – 2000 mg curcumin to study the maximum tolerated dose. The curcumin capsule was inserted intravaginally once daily. However, none of these approaches are for long-term treatment and have not been successfully translated to the clinic.

In all the clinical trials, drugs are administered daily, either in the doctor's office, as in the case of cervical cap, or inserted daily by the patient, as was in the case of curcumin capsule. In both these clinical trials, excess compound was used to deliver the required amount. In our approach, the cervical insert can be placed within the cervix once, and will deliver test drug both to the cervix and the transformation zone, the site of cervical cancer development. Further the drug will be delivered "24/7" for many months or perhaps even a year. The device is fashioned in such a manner that the device can be in place during menstruation. Since the device releases WFA directly in the cervix, we found WFA levels at approximately 10 – 16 ng/g tissue while levels in other organs including uterus and ovary were below detection.

Conclusion

The cervical implant device has great potential and is expected to be a "game changer" in the treatment of women with high-grade cervical dysplasia (CIN2+). The cervical implant can also be utilized to treat vaginal and vulvar pathologies. The delivery of therapeutics directly at the target site eliminates the systemic toxicities associated with conventional oral or parenteral drug formulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cervical polymeric implants delivers plant therapeutics to the target organ, Cervix.
- The polymeric implants release withaferin A (WFA) locally and continuously.
- Coatings with blank polymer reduce burst release of drug.
- Levels of WFA was dose dependent and restricted to local/surrounding tissues.
- Cervical implants do not exhibit any systemic or tissue toxicity.

Figure 1. Development of different models of polymeric cervical implants. **A** - pictures of the prototype polymeric cervical implants. Shown are two different sizes with different views; **B** - graphical design; and **C**, placement of the implant in the cervix. **D** -Different designs of cervical implants tested for suitability in the goats; and **E** shows the device fabricated to hold and insert the implant in the goat cervix.

Figure 2.
Effect of sham polymer coating on withaferin A (WFA) release using small cylindrical implants. Rate of release of withaferin A (WFA) from cylindrical implants (15 mm long $\times$ 2.2 mm dia; 70 mg; 4% WFA load), with and without sham polymer coatings. Data denote mean of 3 implants $\pm$ SD.

Figure 3.

In-vitro and *in-vivo* release of withaferin A (WFA) from cervical polymeric implants. Rate of release of withaferin A (WFA) from cervical implants (2 g; 4% WFA load), with and without sham polymer coatings; inset shows cumulative release. Data denote mean of 3 implants $\pm$ SD. Right panel shows the *in vivo* drug release from the polymeric implants.

Figure 4. Histopathological analysis of goat cervical tissues following withaferin A (WFA) treatment. Histopathological images of the cervical tissue collected from the untreated goats and goats having sham or 4% withaferin A (WFA) implants for 30, 60 and 90 days. Biopsies were collected at the indicated times and Hematoxylin and Eosin (H&E) stained images were analyzed for drug related toxicity. Only representative images with higher WFA (4%) implants are shown. Images were captured at 25X magnification.

Figure 5. Toxicity screening (blood chemistry profile) of withaferin A (WFA) delivered via polymeric implants in goats. At the time of necropsy blood was collected and analyzed using an automated AU640® Chemistry Analyzer by Antech Diagnostics. Data represent average $\pm$ SD of three animals. Statistical analysis was performed by student t-test.

Figure 6.
 MS spectra of withaferin A (WFA) (A) and daughter peaks (B).

Figure 7.

Tissue levels of withaferin A (WFA) from cervical implants. Levels of withaferin A (WFA) in indicated tissues of goats 90 days after receiving 2% and 4% WFA analyzed by LC/MS-MS. Multiple-reaction monitoring (MRM) was used to quantify the WFA and prednisolone was used as IS.