Medroxyprogesterone Acetate Reduced Cellular Proliferation of the Luminal and Glandular Epithelium in the Developing Canine Uterus

Katharyn Brennan, Robyn Wilborn, Meghan Davolt, Anne Wiley, Dori Miller, Paul Cooke, Aime Johnson, Bruce Smith, and Frank Bartol

ABSTRACT

The uterine gland knockout (UGKO) phenotype was produced in both sheep and mice by strategic administration of progestins to neonates from birth (Postnatal Day = PND 0). Adult UGKO animals lack uterine glands and cannot support pregnancy. Induction of the UGKO phenotype in dogs would provide a means of inducing sterility non-surgically. In the dog, uterine gland development begins during the first week of neonatal life and progesterone receptors are present in uterine tissue at this time. The objectives of this study were to determine the effects of neonatal progestin treatment on canine uterine gland development. Seven mixed breed puppies were given either medroxyprogesterone acetate (MPA; n=3) (10mg/kg body weight, i.m.) or an equal volume of sterile saline (n=4) at PND 5 and again once birth weight had tripled. Gland penetration measurements were obtained from uterine cross-sections which were stained with Hematoxylin and imaged using the Aperio® Imaging system. Additionally, uterine cross-sections were stained with POPO-1 to visualize cell nuclei and immunohistochemically stained for cytokeratin 8 (CK8), an epithelial marker, and proliferating cell nuclear antigen (PCNA), a marker of cell proliferation. Primary antibodies were localized using fluorochrome-labeled secondary antibodies in order to produce target-specific signals at defined emission wavelengths. Images were obtained using the Nuance FX® multispectral imaging system and spectrally unmixed images were analyzed using Cell Profiler™ and Cell Profiler Analyst™. Quantitative data were subjected to analyses of variance. No effects of treatment on uterine gland penetration depth were identified. However, a treatment by cell-compartment interaction was detected (P < 0.01) for PCNA labeling index, with the luminal and glandular epithelium experiencing reductions in cell proliferation. While uterine gland development was not completely abolished, MPA significantly reduced cell proliferation within glandular and luminal epithelial cells, indicating that manipulation of uterine development in the canine may be possible with further investigation.

INTRODUCTION

Millions of companion animals are euthanized each year (Olson, 1993) and companion animal overpopulation poses a threat to the quality of life for animals as well as the health of humans (Fournier, 2005). Currently the most effective means of population control is sterilization surgery (Kutzler, 2006). Surgery can be costly, inconvenient, requires specialized and sterilized equipment, and comes with a certain degree of morbidity and, less commonly, mortality. Thus, discovering a method to induce sterility non-surgically in companion animals is an area of great interest.

Uterine gland development, or adenogenesis, in the dog
begins within the first week of life and is complete by 6 weeks of age (Cooke, 2012). During the pre-pubertal time period, there should be no endogenous steroid hormones (i.e., estrogen or progesterone) in circulation. Exposure of neonatal sheep and mice to progesterone or progesterone-like compounds (collectively called progestins) has been successfully used to induce the uterine gland knockout (UGKO) phenotype (Bartol, 1999; Cooke, 2012). Uterine glands secrete critical substances that support fetal growth during pregnancy (Gray, 2001) and adult UGKO animals cannot support pregnancy due to their aglandular phenotype (Bartol, 1999; Cooke, 2012). Developing a UGKO phenotype in the dog would potentially provide the means to induce sterility without the need for surgery.

The presence of progesterone and estrogen receptors have been demonstrated in the canine uterus at seven days of age (when uterine gland development begins in this species) and these receptors continue to be expressed until six weeks of age (Cooke, 2012). Medroxyprogesterone acetate (MPA) is a synthetic progestin that is bioactive in the dog (Beijerink, 2007). Previous research looking at MPA exposure in the female dog has been focused on studying long-term effects of MPA exposure as a means to control reproductive cyclicity in adult females (Von Berky, 1993). However, there is very little information regarding the use of MPA in juvenile dogs. Although previous MPA studies in dogs did not examine neonatal exposure, they did provide critical dosage information to help ensure that deleterious effects were not observed later in the puppies from this experiment (Smith, 1993).

Given that uterine gland development in the dog begins within the first week of life and given that MPA has been shown to act as a bioactive progestin in the dog, the objective of this experiment was to determine the effects of neonatal MPA treatment on uterine gland development and cell proliferation in the canine uterus. It was hypothesized that neonatal MPA treatment during a critical developmental time period would alter normal uterine development and specifically would inhibit the development of uterine glands. Changes in uterine histology with respect to uterine glands and cell proliferation were analyzed to determine the effects of MPA treatment in neonatal canine uterine tissue.

METHODS

Animals, MPA Administration, and Assay

Seven mixed breed puppies from three litters were used in this study. There were three MPA-treated animals and four...
controls. Beginning at PND 5, puppies were given either MPA (10mg/kg body weight, i.m.) or an equal volume of sterile saline. Because neonatal puppies grow at an exponential rate, the MPA dose was repeated once birth weight had tripled (between 2-3 weeks of age) (Figure 1). Following ovariohysterectomy (OHE) at PND 56, puppies were monitored for 10 days under the care of a veterinarian and then adopted out to permanent homes.

Serial blood samples of 1-1.5 mL were obtained via jugular venipuncture on PND 5 prior to treatment (baseline) and then subsequently 14, 20, 28, 42 and 49 days post-treatment to determine serum MPA concentration. Serum samples were stored at -80°C and then serum levels of MPA were measured by a conventional radioimmunoassay (RIA) method using commercial kits (Immunometrics Ltd.; London, UK). Serum samples were diluted with 0.9% NaCl at a ratio of 1:10 prior to assay. The RIA method is an extraction type RIA and utilizes tritium-labeled (3H)-MPA as a tracer and the specific anti-MPA polyclonal antibody produced in New Zealand rabbits. MPA in serum samples was extracted with diethyl ether. The solvent containing the extracted MPA was evaporated to dryness and reconstituted with the assay buffer. The residue was dissolved and incubated with tracer and antibody overnight. Bound and free steroids were separated using dextran-coated charcoal suspension. The linear range for the standard curve was 20 to 1300 pmol/L and the sensitivity of the assay was found to be 70 pmol/L. The assay is monitored using four internal quality control samples (Pools 1, 2, 3 & 4 with MPA concentrations in the range of 900, 1600, 2700 and 5100 pmol/L, respectively) included in each assay. The intra- and inter-assay coefficients of variations were less than 10% in these assays for all four quality control samples used.

**Figure 2.** Uterine cross-sections obtained via OHE on PND 56, stained with hematoxylin and imaged using the Aperio® imaging system. Shown are representative tissues from control (left; panels A, B) and MPA-treated (right; panels C, D) females. Boxes B & D illustrate how data for gland penetration were obtained; note quadrant divisions and the multi-colored lines inside of each quadrant showing measurement sets.

**Tissue Processing & Gland Penetration Measurements**

Uterine tissues were collected at the time of OHE, fixed in 4% paraformaldehyde, and embedded in Paraplast-plus® (VWR International, LLC; Radner, PA). Tissues were sectioned at 6µm, deparaffinized with Hemo-De® (Scientific Safety Solvents; Keller, Texas), mounted
on VWR Superfrost® Plus slides (VWR International, LLC; Radner, PA), rehydrated through a graded series of alcohol rinses to de-ionized water, and stained with hematoxylin. Uterine cross-sections were imaged using the Aperio® Imaging system. Gland penetration depth was determined by measuring gland penetration as a function of stromal compartment depth. Measurements were organized by dividing uterine cross-sections into four quadrants, beginning at the mesometrium and continuing systematically in a clock-wise direction around the tissue section (Figure 2). Two sets of measurements were taken per quadrant with fourteen total sets of measurements obtained per animal. All data were subjected to analyses of variance using GLM procedures (Statistical Analysis Software, SAS; Cary, NC).

Immunohistochemical (IHC) staining

Mounted tissue sections were subjected to heat induced antigen retrieval in a citrate buffer solution (pH 6.0). After cooling, a blocking solution of 10% non-immune goat serum (1:10 v/v; diluted in PBS) was applied at room temperature for 20 minutes in a humidity chamber. Primary antibodies were applied simultaneously and sections were allowed to sit at room temperature for one hour. Anti-proliferating Cell Nuclear Antigen (PCNA), specific for an auxiliary protein of DNA polymerase delta, mouse monoclonal-IgG2a (Santa Cruz Biotechnology; Santa Cruz, CA) was applied at a 1:50 v/v dilution. Guinea Pig polyclonal anti-Cytokeratin 8 (CK8; Fitzgerald Industries Intl; Acton, MA) was applied at a 1:250 v/v dilution. Primary antibodies were diluted in 10% goat serum/PBS solution. AlexaFlour®-labeled fluorescent secondary antibodies (ThermoFisher Scientific; Waltham, MA), A594 (goat anti-mouse IgG2a, 1:400 v/v) and A568 (goat anti-guinea pig CK8, 1:400 v/v) were applied and incubated for one hour. POPO™-1 iodide was applied as a nuclear counterstain (1:10 1mM/200uL, Invitrogen Corporation; Carlsbad, CA) and incubated for 20 minutes at room temperature. Coverslips were mounted using VectaShield® Mounting Medium for fluorescence (Vector Laboratories, INC; Burlingame, CA) and slides were stored overnight in a dark humid chamber at 4°C.

Multispectral Imaging (MSI)

Slides were imaged using the Nuance FX MSI system (PerkinElmer; Hopkinton, MA). Images were obtained at 40X magnification. Image data was collected from 420 – 720 nm at 10 nm wavelength increments.
Acquiring a spectral library allowed the images to be digitally unmixed according to the specific emission wavelengths for each fluorescent label. Spectrally unmixed images were analyzed using CellProfiler™ and Cell Profiler Analyst™ (www.cellprofiler.org) (Figure 3). Data were collected after constructing a CellProfiler™ “pipeline” that could identify nuclei, distinguish PCNA-positive versus PCNA-negative nuclei, and collect data according to cell compartment (luminal epithelium = LE, glandular epithelium = GE, stroma = ST, myometrium = MYO).

RESULTS

When uterine characteristics were compared between control and MPA-treated females, several differences were noted. Cellular arrangements visibly differed between control and MPA-treated females (Figure 2). However, only a few of these parameters were supported by statistical differences. When gland penetration depth was examined, there was no effect of treatment (mean gland penetration depth for control animals was 153.5 ± 46.2 µm and for MPA treated animals was 113.0 ± 12.7 µm).

When the four uterine cell compartments were compared, it was determined that glandular epithelium and luminal epithelium were affected by treatment. A treatment by cell-compartment interaction

Figure 4. PCNA Labeling Index (LI) data for control (blue bars) and MPA-treated (red bars) tissues. Data are expressed as percent PCNA-positive cells for luminal epithelium (LE), glandular epithelium (GE), endometrial stroma (ST), and myometrium (MYO). A treatment by cell compartment interaction (* = P < 0.01) was identified indicating that effects of MPA on reduction of PCNA LI were more pronounced in some cell compartments, as illustrated.

Figure 5. MPA serum concentrations (ng/mL) as a function of age in MPA-treated (red line) as compared to control females (blue line). In treated animals, serum MPA concentrations increased after the first injection at PND 5, reached peak levels one week after the booster injection was given when the birth weight had tripled at PND 25±1, and remained elevated for the duration of the experimental period.
was detected (P < 0.01) for PCNA labeling index, indicating compartment-specific changes in cell proliferation associated with MPA exposure (Figure 4). Specifically, cell proliferation within glandular epithelium and luminal epithelium was significantly reduced in the MPA-treated animals as compared to the controls.

Measurements of MPA concentrations in peripheral circulation confirmed adequate absorption of the drug at the dosage and volume administered. Compared to controls, circulating MPA levels were elevated at the first time point post-treatment and remained elevated through the end of the experimental period in treated animals (Figure 5).

**DISCUSSION**

While differences due to treatment were not as profound as those previously reported in uterine tissue of other species, important changes were observed. In sheep, cattle, and mice, a similar experimental design successfully induced the UGKO phenotype; this model did not achieve the same effect in the dog. In each of the previous UGKO animal models, the critical window for progestin exposure was highly specific and determined within those studies. In the dog, the critical window for progestin exposure has not yet been determined; defining this time period should be explored further.

Due to the exponential growth rate in neonatal puppies, there was some concern that the MPA would be metabolized before their bodies could respond to the treatment. The ability to measure detectable concentrations of MPA in systemic circulation throughout the study was essential to confirm that puppies absorbed the medication and to provide assurance that MPA was in systemic circulation throughout the study period (Figure 5).

The extent to which MPA acts as a progestin in the neonatal canine uterus is unclear. It has been shown to bind promiscuously with glucocorticoid receptors in the adult canine (Selman, 1996). Therefore, it is possible that MPA preferentially bound glucocorticoid receptors and did not elicit as robust a response as if it had bound preferentially with progesterone receptors. Alternatively, it is also possible that glucocorticoid signaling pathways could provide the means to induce a UGKO phenotype in the dog.

Establishing non-surgical methods of sterilization for companion animals has many benefits including reducing morbidity and the high costs associated with OHE surgery. Neonatal progestin exposure as implemented here did not induce a UGKO phenotype, but did reduce endometrial cell proliferation as reflected by labeling index for PCNA (Figure 4). MPA was shown to be anti-proliferative in the neonatal canine uterus and these results align with the observations of UGKO phenotypes in previous studies, just to a lesser extent. Results indicate that uterine gland development in dogs may be abolished with identification of appropriate anti-adenogenic conditions. Alternate signaling pathways such as the glucocorticoid receptor should also be explored to determine if there is a more efficient treatment to accomplish these goals in the female dog.

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**REFERENCES**

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