Theriogenology 95 (2017) 105–112

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Histomorphometrics and quantitative unbiased stereology in canine uteri treated with medroxyprogesterone acetate

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ARTICLE INFO

Article history:
Received 13 May 2016
Received in revised form
13 March 2017
Accepted 13 March 2017
Available online 14 March 2017

Keywords:
Canine
Uterus
Medroxyprogesterone acetate
Progestin
Morphometry

ABSTRACT

This article describes the effects of MPA use on the canine uterus using stereological methods. Entire reproductive tracts were removed from normal healthy canine bitches (*Canis lupus familiaris*) and grouped as: nulliparous (n = 11), multiparous (n = 11) and MPA-treated (n = 11; nulliparous; two treatments; 5 mg/kg). 1 cm samples were cut from the corpus, horn and uterine tube and fixed in 10% formaldehyde. Sections of each were mounted on slides and stained with hematoxylin-eosin. We assessed the fraction area for components of endometrium and myometrium and V_V (volume density) and S_V (surface density) of the gland and stroma using the M_{36} test system provided by the STEPanizer Stereological Tool. No gross histological differences were observed between study groups in the uterine tube, uterine corpus and horn. The wall of the uterine corpus and horn in MPA-treated bitches was characterized as being thicker than in the other groups. A cross-section of the uterine corpus revealed no differences between components of uterine wall in the corpus and horn; however, differences were observed in the volume density [V_V; %] in variables such as: $V_{V[str.vasc/uterus]}$ (nulliparous *vs.* multiparous; $p = 0.0019$) and $V_{V[str.supravasc/uterus]}$ (multiparous *vs.* nulliparous and MPA; $p = 0.0035$). In the endometrial gland, differences were detected in $S_{V[gland/endom]}$ (multiparous *vs.* MPA, $p = 0.0442$). In the uterine horn, differences were only observed in the variable $V_{V[lumen.gland/endom]}$ (multiparous *vs.* MPA; $p = 0.0019$). This study shows quantitative changes in the architecture of the endometrium and myometrium in all the uterine segments, mainly morphological endometrial gland changes of the uterine corpus, increasing the surface area per unit of volume; however, these changes usually do not differ quantitatively from those observed in the uterus of multiparous bitches.

1. Introduction

Hundreds of dogs are euthanized annually under public health programs in order to control the overpopulation of stray dogs in urban centers. The usual method, surgical sterilization [1], prevents and reduces the frequency of uterine and mammary gland diseases. However, due to the high costs associated with performing a large number of surgical procedures and the possible postoperative complications, non-surgical methods have been proposed. One of these is treatment with medroxyprogesterone acetate (MPA), a long-acting synthetic progestin. In the United States, its use is not approved as a contraceptive treatment in dogs; however, its use in other countries to delay estrus is frequent. Although its effect is similar to luteal endogenous progesterone, a high incidence of side effects has been reported, including adrenocortical atrophy, diabetes mellitus, acromegaly and pyometra, among others [2–4]. In dogs treated with MPA for estrus suppression, the prevalence of clinical presentation of uterine and mammary disease is 45% [5]. It is estimated that the risks of MPA-related side effects are common and should be considered prior to the choice of surgical or non-surgical castration [1,6]. On the other hand, studies have reported normal morphology of the uterine tissue. For example, one study [7] evaluated the morphological and proliferative changes in the uterine tubes during anestrus, the luteal and follicular phases and the morphometric characteristics of the tubular epithelium. Changes in the endometrial epithelium have also been described in

Abbreviations: MPA, Acetate of Medroxyprogesterone; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; V_V, density volume; S_V, Surface density; SD, standard deviation.

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http://dx.doi.org/10.1016/j.theriogenology.2017.03.008
0093-691X/

bitches in anestrus and metestrus [8] as well as the influence of steroid hormones on the histological characteristics of the uterus [9]. Previous research looking at MPA exposure in the female dog has focused on studying the long-term effects of MPA exposure as a means of controlling reproductive cyclicity in adult females [5], its effects on adenohypophyseal function [3] and pulsatile plasma profiles of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) before and during MPA treatment [4]. However, there is insufficient information regarding the application of MPA during anestrus and its quantitative effects on uterine tissue.

The quantitative investigation of images taken from light microscopy observation is one of the pillars of biological and biomedical investigation. The introduction of stereology and design-based sampling in the biomedical field has been a major advance over the last 30 years [10]. The main advantage of this technique is that it can efficiently produce reliable and accurate results. Stereological estimates such as volume density (V_V) and surface (S_V) in uteri of bitches treated with MPA during anestrus have not been assessed previously; therefore, the interpretation of the results obtained here was difficult due to the absence of this subject in the literature.

It was hypothesized that intramuscular MPA treatment during anestrus would modify the uterine tissue and specifically would affect the development of components of the endometrium and myometrium as well as the morphology of the uterine tube. The purpose of this study was to develop a base of metric information regarding the effects of intramuscular MPA administration on the uterus and uterine tube. Specific objectives were to describe the quantitative histomorphological and unbiased stereological features in the uterus of female dogs exposed to the synthetic progestin MPA and to compare them with females exposed to physiological luteal progesterone under conditions of nulliparity and multiparity.

2. Materials and methods

This study was approved by the competent university review boards, and was conducted at the Center for Excellence in Surgical and Morphological Studies of the Universidad de La Frontera, Temuco, Chile.

2.1. Animals, samples and determination of estrus cycle

Complete uteri were obtained from 33 healthy adult female dogs (*Canis lupus familiaris*) from the University's Animal Hospital, with no defined breed, subjected to ovariohysterectomy during anestrus (18 months - 6 years). No gross abnormalities were present anywhere in these tracts. Uteri were classified as nulliparous (n = 11; older than 9 months, that had experienced at least one estrus cycle), multiparous (n = 11; older than 9 months, that had experienced at least two full-term gestations and involution of the uterus after the last gestation was complete) and MPA-treated bitches (n = 11; nulliparous exposed twice to contraceptive treatment during late anestrus). All the bitches included in this study demonstrated at least one estrus, with this information being determined from the owner's report and clinical records (Table 1). Treatment with a synthetic progestin depot preparation of MPA (OVO-6® 50 mg, Drag Pharma Laboratory Invetec S.A., Chile) was begun during anestrus giving two treatments of 5 mg/kg i.m. body weight at 8-week intervals. A vaginal inspection and cytological examination of the vaginal floor were performed on bitches used in this study to determine the estrus cycle and confirm the anestrus. Three days before the start of the treatment with MPA the stage of the estrus cycle was assessed. Anestrus was confirmed using physical examination, vaginal cytology (Diff Quick®, Hartman Leddon Co, Philadelphia, PA), histological parameters of the uterus and ovaries [11] and confirmed by the absence of the corpus luteum in the ovary. Vaginal cytology showed sparse numbers of parabasal cells and variable numbers of neutrophils. The vaginal mucosa appeared thin and red with visible capillaries; the surface was easily traumatized and vaginal cytology was difficult to monitor without inducing bleeding with spurious erythrocytes in smears classified as being in anestrus [12]. In terms of uterus histological parameters, bitches in anestrus had simple low columnar or cuboidal epithelium, superficial stroma rich in cells, and the stroma of the deeper part of the endometrium with less convoluted basal glands with simple cuboidal and in some cases columnar epithelium.

2.2. Uterus volume measurement and tissue preparation

The fresh uteri were separated from the ovaries and uterine tube. The broad ligament was dissected. The volume of each uterus was measured by the immersion method (Scherle 1970). Three 1 cm sections of each uterus (corpus and horn) and uterine tube (ampulla) were cut and fixed in 10% formaldehyde (Fig. 1A). The samples were processed to obtain paraplast sections for microscopic evaluation (Paraplast Plus embedding medium; melting point: 54 °C; Sigma-Aldrich Chemical Co., St Louis, MO, USA). The sections of uterus were processed through a series of alcohols with increasing concentration and cleared in xylene. The paraplast block was cooled and stored at 4 °C until sectioning. Sections (5 μm) deparaffinized and rehydrated through decreasing alcoholic solutions were mounted on slides. Sections of each sample were routinely stained with hematoxylin and eosin.

2.3. Morphometric, planimetric and stereological analysis

Digital photomicrographs of each tissue sample were obtained using a Leica®DM750 optical microscope equipped with a Leica®MC170HD digital camera. A histological evaluation at 40× was performed on uterine tube to describe the condition of the three groups evaluated. For histomorphometry the cross-histological sectional images of uterine tubes, horns and uterine corpora were measured using the AxioVision 4.7.1 software (Carl Zeiss, Jena, Germany). For planimetry, the fraction area of the uterine wall components (corpus and horn) was calculated by light microscopy at 1×, 2× and 4× magnifications (%; fraction area of endometrium, submucosal, vascular and supravascular layers). For stereology, sampling and acquisition of unbiased stereological estimators were performed. Serial histological sections (20 μm intervals) 5 μm thick were analyzed (corpus and horn); each stereological data was estimated by examining 5 microscopic fields per sample [13]. In the endometrium, the volume density (V_V; estimated by point counting) of the stromal tissue and lumen and glandular epithelium as well as surface densities (S_V; estimated by intersection counting) of the endometrial gland were calculated by light microscopy at 40× (Fig. 1B). The M_{36} test system provided by the STEPanizer Stereological Tool software [14] was used, which has 36 test points; the test line measures 18d and the test area measures 36.36d^2. The overlapping program analyzed images from the horns and uterine corpora. Total volumes of glandular epithelium, lumen and stroma within the endometrium of each uterus were determined by multiplying the mean proportion of each tissue within each uterus by the total volume of each respective uterus [15]. All stereological and planimetric evaluations considered the tissue deformation [16]. All measurements were performed by two trained observers, each using his own microscope and computer software, blinded to the identity of the groups.