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Morphological, histological and molecular investigations on canine uterine tissue after ovariectomy

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ABSTRACT

In the present study, we investigated the course of atrophy in canine uterine tissue and the expression of estrogen receptors (ER) and progesterone receptors (PR) within 6 months after ovariectomy (OE). In nine primipar bitches of different breeds, bilateral OE and removal of one horn was performed. Six months after surgery, the remaining uterine tissue was removed. The tissue was examined for signs of inflammation and proliferation, and for expression of ER, PR and Ki67 by means of immunohistochemistry (IHC); furthermore transcription of *vascular endothelial growth factor* (*VEGF*), *transforming growth factor-ß* (*TGF-ß*), *epithelial growth factor* (*EGF*), *platelet activating factor* (*PAF*), *tumor necrosis factor α* (*TNFα*), and their specific receptors was determined by means of RT-qPCR. Serum concentrations of estrogen and progesterone were measured immediately before the first and second operation. Six month after OE, no inflammation was seen in any uterine tissue, the thickness of the stump was decreased in most bitches. Protein expression of Ki67 revealed high individual differences after the second operation. Concentration of both hormones was not significantly changed, the estrogen concentration always revealed high individual differences. The expression of ER was significantly decreased in stromal and smooth muscle cells of the uterine tissue ($p < 0.01$), and the expression of PR in stromal cells only ($p < 0.05$). The gene expression of growth factors did not change significantly between first and second operation. We conclude that complete atrophy did not occur within 6 months after OE, instead, a high percentage of uterine cells still expressed ER and PR, rendering the stump susceptible to hormone treatments.

1. Introduction

Surgical castration of animals is a common procedure in small animal veterinary medicine. For castration of female dogs, many different techniques have been described in the literature. They all include the removal of the ovaries and a varying portion of the uterus. During ovariohysterectomy (OHE) the complete cervical mucus membrane is removed and no reactive tissue remains. This procedure is the method of choice for most uterine diseases. In most countries, bilateral OE is a common alternative to traditional OHE [1] and especially endoscopical ovariectomy meanwhile is a common technique [2–6]. Conventional OE was not found to be quicker than OHE [7], however, when endoscopically employed, the procedure was quicker and less post operative complications were observed than after conventional OHE [4]. This procedure would therefore be preferable for any routine castration [8,9]. During the past decade, few studies were performed, comparing the clinical effects of both surgical methods. Regarding the incidence of side effects observed by the owners, no statistical difference between OHE and OE was found in long term observation studies [1,10]. In one study, the incidence of vaginal discharge and urinary incontinence was compared about 8–11 years after OE and OHE, respectively; differences between groups were not significant [1].

It has been mentioned that dogs or cats that have undergone OE could develop cystic endometrial hyperplasia-pyometra (CEH) should they ever come under the influence of progestins [9,11].

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Furthermore [12], reported a uterine stump empyema in a castrated bitch after 3.5 years of daily estriolum application. They concluded that the estrogenic effects like proliferation and edema of the cervix might have triggered the infection. In another study, castrated bitches received ostriol tablets for 42 days and some showed mild bloody vaginal discharge [13].

However, it is not known, whether and how long the comparably large uterine stump remaining after OE expresses reactive hormone receptors or factors capable to initiate proliferative activity or inflammation. To the best of our knowledge, there is no controlled study describing the morphological, histological and molecular changes of the uterine stump after OE.

Aim of the study was thus to investigate whether uterine tissue, remaining in the bitch for 6 months after OE, still expresses hormone receptors, or factors capable to initiate proliferative activity or inflammation. Hypothesis was that within 6 months after OE, uterine tissue undergoes complete atrophy without signs of inflammation or proliferation.

2. Animals and methods

2.1. Animals

Nine primipar bitches of different breeds (five mongrels, one Anatolian Shepherd, one German Shepherd, one terrier and one Great Dane) aged 2–5 years with a body weight of 15–20 kg were included into the study. The bitches were kept in private households and had a history of regular sexual cycles.

2.2. Ethical approval

The experiment was conducted in the Adnan Menderes University, Dept. of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Aydin (Tr). The animal experimental protocol for the study was approved by the Local Animal Ethical Committee (Adnan Menderes University Ethical Committee Number: 64583101/2016/94). All owners gave their written consent, that the bitches may be bilaterally castrated with one uterine horn remaining in the abdomen, and may undergo a second operation after 6 months.

2.3. Operations and sample collection

All bitches were examined clinically and gynecologically at the beginning of the study. At 4 months after the end of estrus, the presence of anestrus was assessed by means of vaginal inspection, cytology and progesterone (P4) measurement. Before the operation, a blood samples was examined for blood glucose concentration, blood urea nitrogen (BUN), blood albumin, packed cell volume (PCV), leukocyte and total plasma protein concentrations; all values were within the range of normal for all dogs. Serum was stored for measurement of P4 and estradiol 17β (E2) concentrations at −80 °C until further processing. Thereafter, bilateral ovariectomy and unilateral hysterectomy were performed under general anaesthesia. After surgery, bitches were presented to the clinic every two months and a clinical examination, blood count and ultrasonography of the abdomen were performed.

The second operation was performed six months after the first; before the operation, all bitches were examined again and a blood sample was taken for the same analyses as before. Tissue samples of the uterine horns were taken immediately after each surgery as follows: a piece of 2 × 2 cm (perimetrium, myometrium, and endometrium) was excised from three different sites of the uterine horn (anterior, middle and posterior third). One half of each piece was fixed in 4% buffered formaldehyde and embedded in paraffin. Histological sections were Haematoxylin and Eosin (H&E) stained and evaluated for signs of inflammation and proliferation. The other half was snap frozen and stored in liquid nitrogen after embedding in Tissue Tec® (Sanova Pharma GmbH, Vienna, A). All samples were sent to the University of Veterinary Medicine, Vienna, Austria, where uterine tissue was investigated by RT-qPCR and histologically.

2.4. Hormone analyses

For hormone measurements, frozen serum samples were thawed at room temperature, and analyses of P4- and E2-concentrations were carried out by Electrochemiluminescence Immunoassay (ECLIA) in an internationally accredited private laboratory (Düzen Laboratory Group, Ankara, Turkey; TURKAK TS EN ISO/IEC 17025:2005), as described before [14]. Extraction efficiency was >95%. The mean intra- and inter-assay coefficients of variance for P4 were 3.2% and 1.7%, respectively. The analytic sensitivity was 0.03 ng/ml. Values for E2 assay were 5.0 pg/ml (sensitivity), 3.0% (intra-assay coefficient) and 1.7% (inter-assay coefficient).

2.5. Immunohistochemistry

Histological sections of canine uteri were investigated by means of immunohistochemistry for the localization and quantity of hormone receptors (PR, ER) and proliferation marker (Ki67). Indirect immunohistochemistry was performed as described previously [15]. Antibodies used were monoclonal anti-progesterone receptor, clone 10A9, Immunotech, Marseille, France, dilution 1:200; polyclonal anti-estrogen receptor Invitrogen, Camarillo, CA, USA, dilution 1:200; monoclonal anti-Ki67, clone 7B11, Invitrogen, Camarillo, CA, USA; all were already positively tested on canine tissue. Epitope retrieval was done by heating sections 4 × 5min in 0.01 M citrate buffer. Evaluation of the sections was performed using a light microscope (Polyvar, Reichert-Jung, Vienna, Austria) with a Nikon DS-Fi1 digital camera and NIS-Elements software. Per slide, three different regions were chosen and 100 cells per region were evaluated and a percentage of positively stained cells were calculated; superficial epithelium, glandular epithelium, stromal cells and muscle cells were evaluated separately. Crypt and basal glands were not evaluated separately. Negative control sections were prepared by omitting the primary antibodies and incubation with PBS.

2.6. Real time RT-PCR

The following factors indicative for proliferation and angiogenesis were investigated by real time RT-PCR with species-specific primers: *vascular endothelial growth factor* (*VEGF*), *transforming growth factor-ß* (*TGF-ß*), *epithelial growth factor* (*EGF*), *platelet activating factor* (*PAF*), *tumor necrosis factorα* (*TNFα*), and their specific receptors. For this purpose, cross sectional segments of the frozen uterine tissue samples (approx. 30 mg pieces) were homogenized with 1 ml of TRI Reagent (T 9424, Sigma-Aldrich, A). After isolation of mRNA, aliquots of the samples were stored at −80 °C until analysis. Isolation of mRNA, quality assessment of the isolated RNA electrophoresis as well as the sequencing procedure was performed as previously described [16,17]. Only samples with a RIN value of >5 were included in the study. Real-time RT-PCR methodology was used as previously described [16–19]. Primers were designed using the Primer Express software 2.0 and 3.0 (Applied Biosystems, Foster City, USA) and Nucleotide blast (NCBI; canis familiaris); all primers and methods have been established previously ([16,20,21,22], Table 1). The probes were obtained from Microsynth (Balgach, CH) und Invitrogen (Salzburg, A). Transcription values were normalized to those of two reference genes, namely β-ACTIN and GAPDH