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CYTOLOGICAL FEATURES OF THE NORMAL ENDOMETRIUM AND OVARIES IN THE QUEEN

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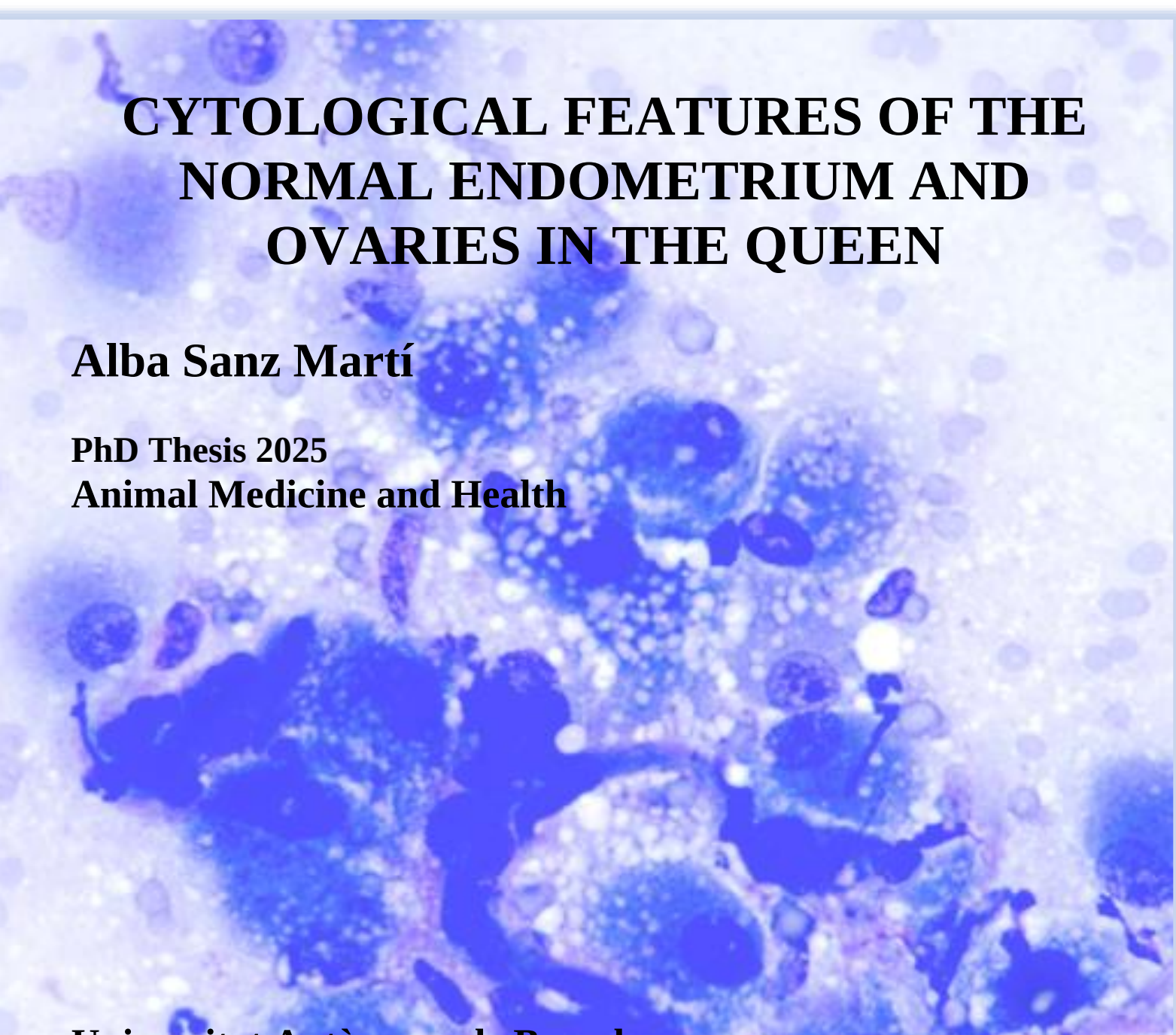
PhD Thesis 2025
Animal Medicine and Health



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CERTIFIQUEN:

Que la tesi doctoral presentada per la doctoranda Alba Martí Sanz, titulada "***Cytological features of the normal endometrium and ovaries in the queen***" per a optar al títol de Doctora per la Universitat Autònoma de Barcelona, s'ha dut a terme sota la nostra direcció. Així mateix, la considerem acabada i apta per a la seva defensa davant de la Comissió corresponent.
I per a què així consti, a efectes oportuns, signem el present document.

Mª Montserrat Rivera del Alamo

Josep Pastor Milan

Bellaterra, Maig de 2025.

CONTENTS

I. ACKNOWLEDGEMENTS

Probablemente, esta es una de las partes más difíciles de escribir y una de las más importantes, puesto que han sido muchas las personas que me han acompañado y ayudado a evolucionar como persona y como profesional, sin las cuales muy probablemente, no hubiera podido llegar a escribir ni la tesis doctoral ni mi trayectoria profesional.

En primer lugar, me encantaría agradecer a mi madre que desde pequeña me enseñó a vivir y disfrutar de la vida y me enseñó que con esfuerzo y perseverancia puedes llegar a alcanzar la luna. Siempre estás y estarás en cada paso que doy. Por supuesto agradecer a mi abuela y a mi tía-abuela su apoyo incondicional, su paciencia, su amor y la entereza y valentía con la que, especialmente en los momentos más complicados, han sabido coger el timón para transformar las situaciones y calmar las tormentas. Sin ellas puedo decir que seguro no estaría escribiendo estas palabras. Agradecer también a mis tíos que han subido y bajado conmigo en la montaña rusa de vivencias y experiencias, que me han ayudado a crecer y a madurar de manera exprés y me han transformado en una persona más valiente y luchadora. A mi abuelo, el más orgulloso que, sin entender mucho hacia donde se dirigía mi trayectoria profesional siempre, creyó en mí de manera incondicional.

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A todas aquellas personas que me han acompañado durante estos últimos años de formación y, en especial al servicio de hematología clínica veterinaria: a Montse, a Rafaela y a Ana, por ser compañeras y amigas. También a Laia que fue una de mis mentoras en mi residencia y que desde el principio creyó en mí y me respaldó.

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II. SUMMARY

Cats are very popular domestic animals, and their breeding is increasing in popularity, especially in the Western World. Currently, the incidence of infertility in the queen is unknown. Some studies conducted in purebred cats indicate that approximately 15% of mated queens do not achieve pregnancy, and there may be significant differences among breeds.

Cytology is a widely used diagnostic technique in both human and veterinary medicine and has become an essential tool in diagnosing infertility in species like mares and cows. However, its use is not so common in small animal species.

The aim of this PhD was to determine the specific cytological features of the normal endometrium and ovaries in the queen to increase the knowledge in this area as a basis to potentially identify pathological processes that may induce infertility.

With this proposal, two different experimental studies were performed:

In the first study, uterine specimens from fifty healthy queens were used to compare two cytological techniques: uterine swab (US) and uterine lavage (UL). Twenty-eight females were included in the uterine lavage group, and twenty-two were included in the uterine swabbing group. The usefulness of the two techniques was compared by evaluating different parameters, including cellularity, background and cell preservation, among others. In an attempt to determine the utility of cytology to detect endometritis, the inflammatory cells admixed with the uterine tissue were also assessed. The results were compared with histopathological and microbiological results. Furthermore, serum levels of progesterone were used to establish the hormonal status of the endometrium. The results obtained showed that both UL and US sampling methods effectively collected enough endometrial cells for diagnosis. However, UL samples showed better correlation with histology. In contrast, US samples had more red and white blood cells, likely due to swab friction causing minor bleeding, which increased WBC levels and RBC/endometrial cell ratios. None of the queens included in the study had endometritis. The results obtained align with data from mares, especially on day 7 of pregnancy, showing greater glandular diameter, epithelial height, and lumen size changes. Those changes are likely associated with early pregnancy and the secretion of uterine fluids that support embryo development.

In the second study, ovarian samples from twenty-four queens were obtained by FNA cytology, and the results were compared with the histological features from the opposite ovary. Furthermore, serum levels of progesterone (Immulite; Siemens Healthcare Diagnostics, Cornellà del Llobregat, Spain) and estradiol (Demeditec Diagnostics Gmbh, Kiel, Germany) were obtained from the included queens to determine the phase

of the reproductive cycle. Cytological features were compared to characterise and describe the cytological features of the ovaries along the reproductive cycle in the healthy queen. Results showed that granulosa cells were present in all cytological samples throughout the reproductive cycle, whereas luteal cells appeared only during the corpus luteum (CL) phase; this finding being consistent with those previously observed in dogs. Unlike previous canine studies, queens in the follicular phase showed fewer mesenchymal cells. These cells have been suggested to play a role in tissue repair and granulosa cell regeneration. Mitotic figures, likely proliferating granulosa cells, were present only in the follicular and anoestrus phases, especially in the follicular phase. Large multinucleated cells were unexpectedly found in all groups, possibly representing apoptotic granulosa cells. Leukocyte numbers generally correlated with blood content in smears, but some CL-phase queens had notably higher leukocyte presence and occasional luteal emperipolesis. This finding was previously reported in dogs and suggests a possible role in ovarian immune regulation.

Knowledge of the specific cytological features of the normal feline reproductive tract is essential for the accurate identification of pathologic processes. In conclusion, cytology may be a valuable tool to detect pathological processes and to improve our understanding of feline reproductive health.

III. ABBREVIATIONS AND SYMBOLS

BP: Baird Parker

CEEAH: Ethical Committee of Animal Care and Research

CL: Corpus Luteum

FNA: Fine Needle Aspirate

FSH: follicle-stimulating hormone

G: Gauge

HPF: High Power Field

LH: Luteinizing Hormone

LPF: Low Power Field

MK: McConkey

MRS: Man Rogosa Sharpe

MSC: Mesenchymal Stem Cell

PBS: Phosphate-Buffered Saline

PMN: Polymorphonuclear Neutrophils

RBC: Red Blood Cell

TSN: Tryptone Sulfite Neomycin

UAB: Universitat Autònoma de Barcelona

UL: Uterine Lavage

US: Uterine Swabbing

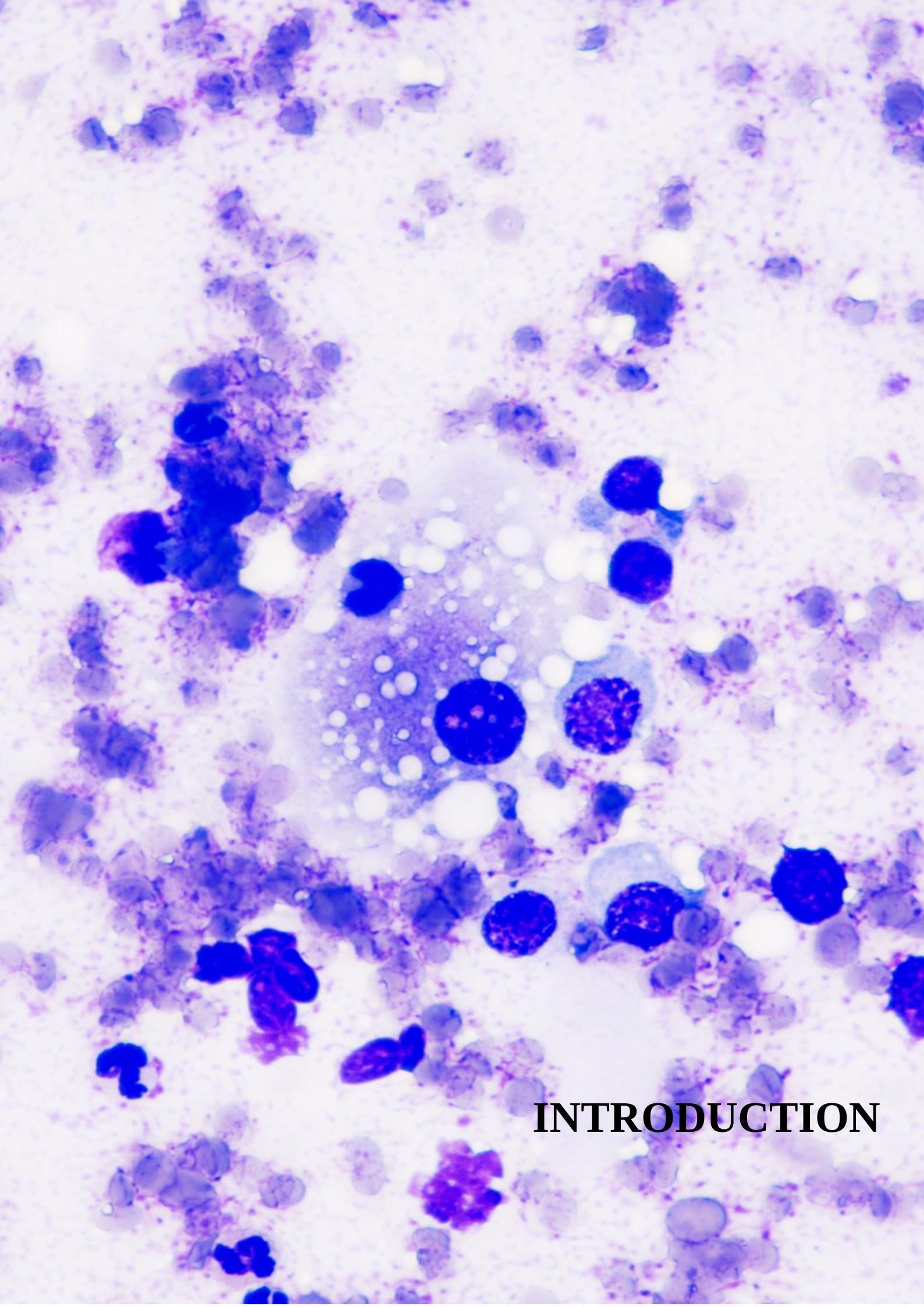
USA: United States of America

WHO: World Health Organization

IV. LIST OF FIGURES AND TABLES

MANUSCRIPT II

Cytological characteristics of the feline ovary at different sexual stages.



INTRODUCTION

V. INTRODUCTION

1.1.-Background

Cytology is an applicable diagnostic tool increasingly used in veterinary medicine to study specimens from a wide variety of animals, including domestic, exotic and laboratory animals, as the most commonly involved (Sharkey & Wellman, 2011). It consists of the morphological examination of the cells from a specific tissue, usually allowing the clinician to reach a rapid approach to the patient, so treatments or clinically relevant decisions can be less time-consuming. Furthermore, in some cases, it also represents a histologically complementary diagnostic tool. Besides being a helpful and fast technique, other advantages that make cytology an interesting diagnostic service are its easy performance, low economic cost and low invasiveness (Marrinhas et al., 2022).

1.2.- History of cytology

Cytology is an ancient technique (Figure 1). It was born in 1660-1665, just after the invention of the microscope. In that period, the cell was discovered as the forming unit of living beings and the first cytological examinations were performed. However, it was not until many years later, at the beginning of the 19th century, that cytology began to be used for diagnostic purposes. Before that time, cytology had a dark period as there were various famous pathologists like Giovanni Battista Morgagni (1682-1771), Matthew Baillie (1761-1823) or Marie François Xavier Bichat (1771-1802), who rejected the use of the microscope (Durdu, 2019).

The first cytological examinations were performed in 1838 by the German pathologist Johannes Müller, who examined tumoural cells in samples taken from neoplastic masses after surgical excision. During the 19th century, microscopical examinations began to be constantly and independently registered and, at the beginning of the 20th century, many types of cancer and neoplastic cells were described (Durdu, 2019).

The development and expansion of cytology as a clinical test in human medicine began with the publications by George N. Papanicolaou and Herbert F. Traut, in 1941 and 1943 respectively, that fueled the use of vaginal cytology as a test to detect uterus carcinoma. During this same period, cytological examination began to be applied to detect cancer in samples from other organs such as the digestive tract, respiratory tract or the central nervous system, among others (Ramzy & Herbert, 2017).

Although in the beginning, many pathologists felt skepticism towards the value of cytology in detecting cancer, after the publication of Papanicolaou's works and during the following decades, cytology was generally developed, and cytopathology became a consolidated discipline. In 1961, Leopold G. Koss and Grace R. Durfee published the book *Diagnostic Cytology and its Histopathological Bases*, which represented an essential tool for those pathologists interested in cytology and that is still today a reference book with the fifth edition published in 2005 (Ramzy & Herbert, 2017).

The growing experience of pathologists together with the development of other diagnostic techniques such as ultrasound, computed tomography or flexible endoscopy, currently allows the assessment of a wide range of body sites, including deep organs, and the study of different diseases such as cancer, infectious diseases and other inflammatory conditions.

In veterinary medicine, cytology history is much shorter compared with human medicine. In spite, in the last decades, it has become widely popular thanks to the rapid obtention of results avoiding the use of more invasive and expensive techniques (Marrinhas et al., 2022).

Both human and veterinary cytology have developed exponentially, and aside from being a rapid preliminary diagnostic test, it also helps to select representative samples to perform other tests that require fresh and non-fixed cellular material like flow cytometry, immunocytochemistry, molecular biology or microbiologic culture (Ramzy & Herbert, 2017).

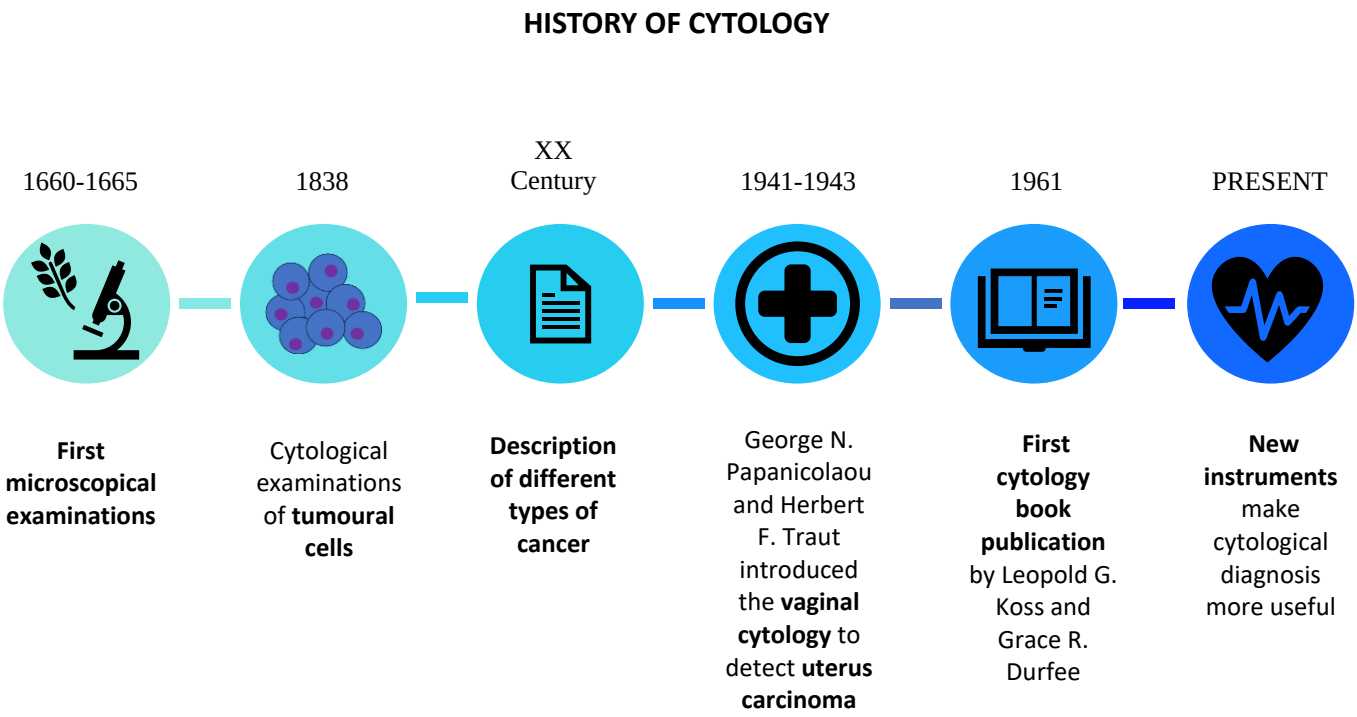


Figure 1. The history of cytology.

1.3.- Cytology in veterinary medicine

As previously described, cytology has had a remarkable evolution during the last years, and it is currently an extended diagnostic tool in the general practice (Marrinhas et al., 2022). Although the primary goal of cytology is to achieve a definitive diagnosis on a specific lesion, in some cases, the sample or type of lesion does not allow to achieve it, but it can help to direct the lesion as an inflammatory, hyperplastic or a neoplastic process (Cian and Monti, 2019; Raskin et al., 2023).

Inflammatory lesions should be classified according to the predominant cell type, and they should be examined carefully for the presence of infectious agents (bacteria, fungi, protozoa). Bacteria generally elicit neutrophilic inflammation, and organisms can be found free in the background and/or phagocytosed by neutrophils (Figure 2). Noninfectious causes that can elicit neutrophilic inflammation include trauma or irritation, tissue necrosis and immune-mediated processes (Cian and Monti, 2019; Raskin et al., 2023).

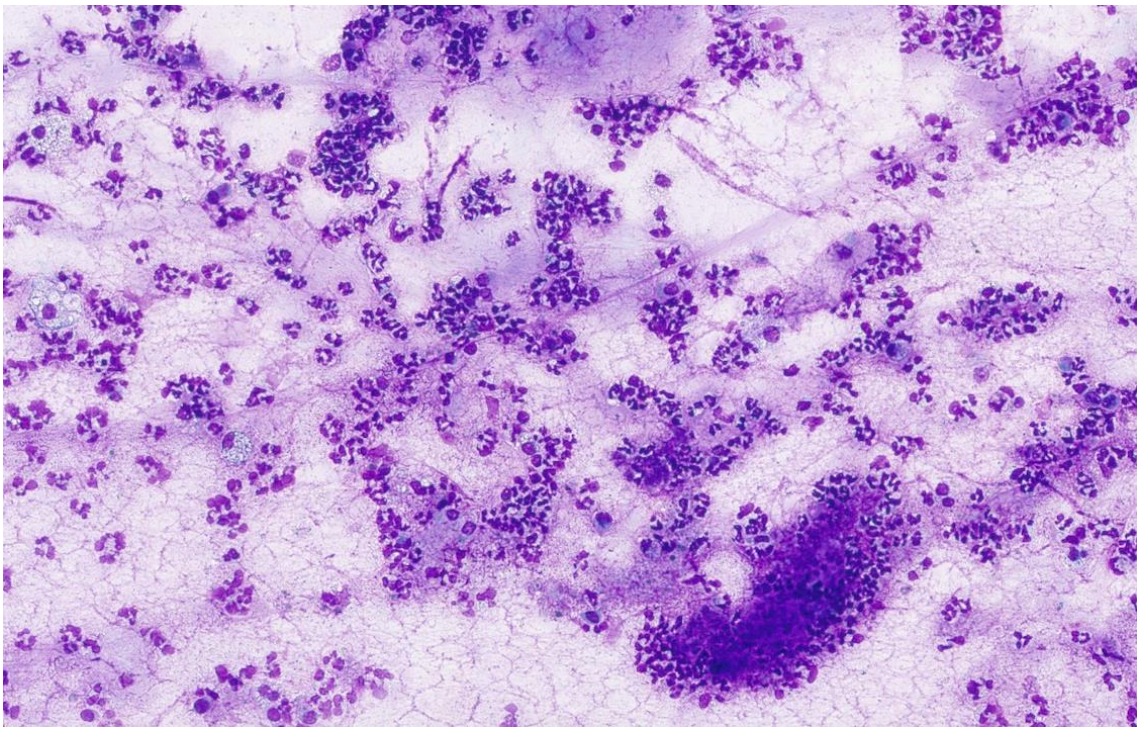


Figure 2: Horse. Bronchoalveolar lavage with evidence of marked neutrophilic inflammation. Neutrophils are degenerated, suggesting septic pneumonia (20x).

Macrophagic inflammation can be caused by selected infectious agents (e.g *Mycobacterium* spp., *Leishmania* spp., fungi, feline infectious peritonitis (FIP) infection), but also noninfectious causes like foreign body reaction or chronic irritation (Figures 3: A and B) (Cian and Monti, 2019; Raskin et al., 2023).

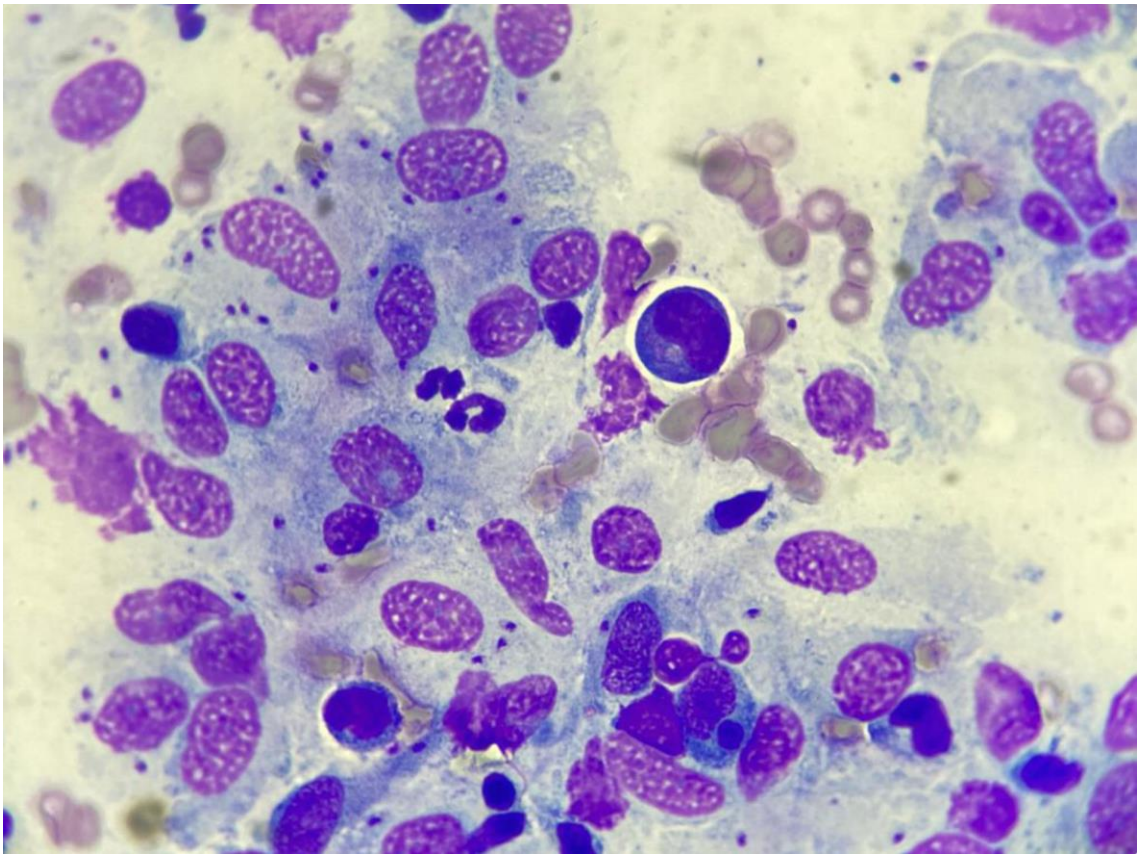


Figure 3 A: Dog. Macrophagic inflammation in the bone marrow of a patient with Leishmania spp. Note the Leishmania spp. amastigotes inside the macrophages and free in the background. One of the macrophages displays erythroid precursor phagocytosis, suggesting an immune-mediated hemolytic component (100x).

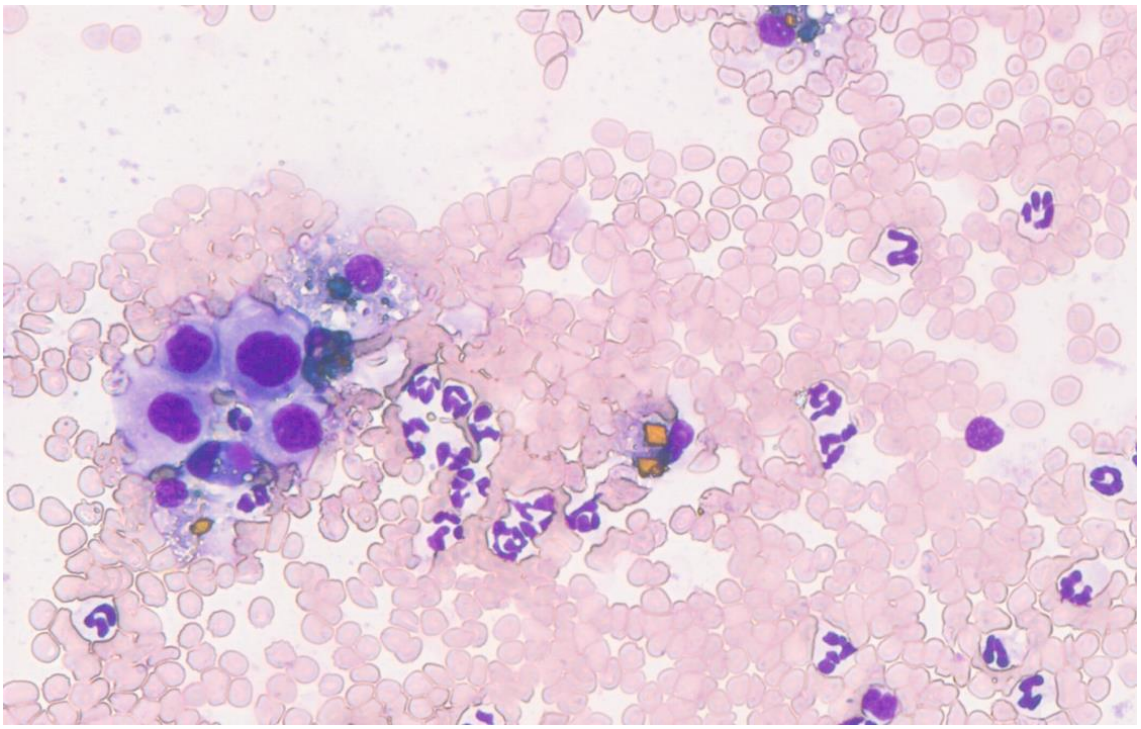


Figure 3 B: Dog, hemopericardium. Neutrophilic-macrophagic inflammation. Neutrophils appear non-degenerate, and there are frequent activated macrophages with signs of phagocytosis. Macrophages contain blue pigment consistent with hemosiderin and sporadic rhomboid crystals compatible with hematoidin crystals. There are also occasional reactive mesothelial cells (40x).

Eosinophilic inflammation is considered when the sample contains a significant number of eosinophils (>10%). Eosinophils are relevant for regulating acute hypersensitivity reactions (type I hypersensitivity) and are commonly observed in allergic conditions and parasitic or fungal infections. Furthermore, they are typically also present as paraneoplastic inflammation in tumours such as mast cell tumours or certain types of lymphomas (Cian and Monti, 2019; Raskin et al., 2023) (Figure 4).

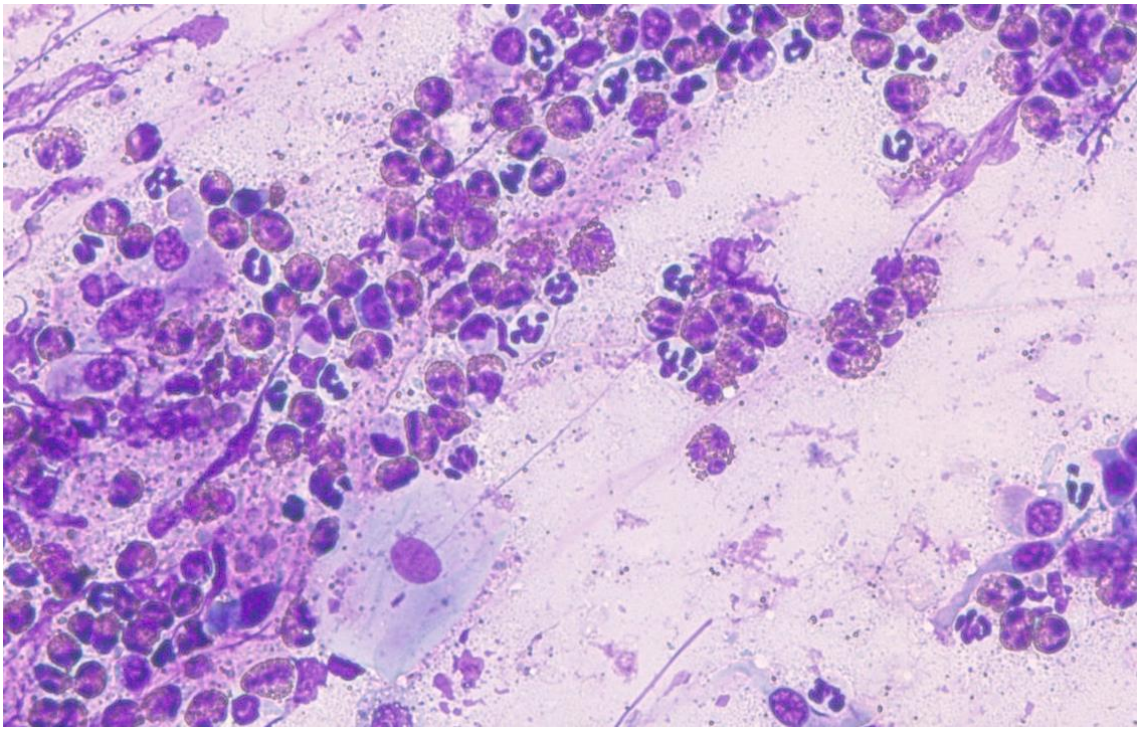


Figure 4: Cat. Bronchoalveolar lavage with evidence of marked eosinophilic inflammation in a patient with allergic bronchitis. In a lower proportion, there are also non-degenerate neutrophils. Scattered oropharyngeal cells are also present (40x).

Lymphocytic inflammation is commonly detected in chronic inflammatory processes, besides viral infections, vaccine reactions, insect-bite reactions, or associated with neoplastic cells, like in regressing histiocytoma (Cian and Monti, 2019; Raskin et al., 2023) (Figure 5).

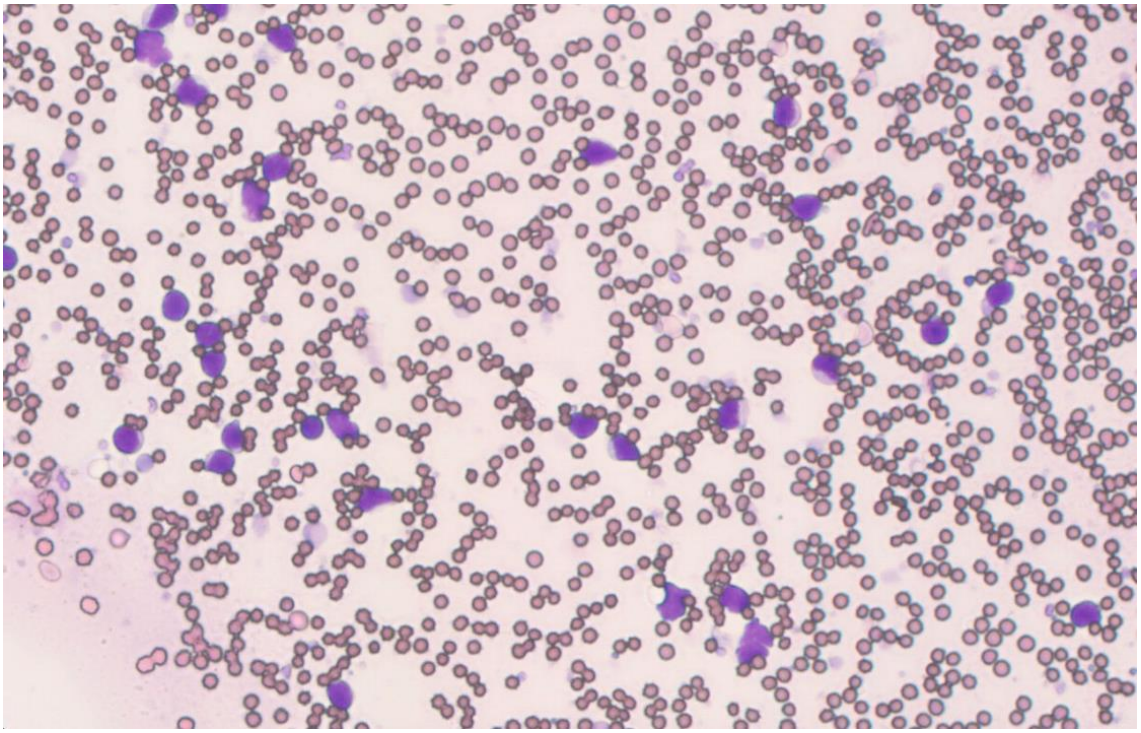


Figure 5: Cat. Lymphocytic inflammation. Numerous small lymphocytes are detected in a hemodiluted background (20x).

Neoplastic or hyperplastic processes are those lesions where tissular cells predominate. Tissue cells can be divided into round, epithelial or mesenchymal based on their morphological features and the type of arrangement (Figure 6). Round cells exfoliate individually and, as their name indicates, they have a round shape with defined cell margins. Epithelial cells often exfoliate in cohesive clusters, and the cell shape can vary from round to cuboidal, polygonal or columnar. Finally, mesenchymal cells are mainly spindle or fusiform and can be arranged individually or in non-cohesive groups. (Cian and Monti, 2019; Raskin et al., 2023). Furthermore, a fourth group belongs to endocrine/neuroendocrine tumours. Those neoplasms originate from secretory epithelial cells (such as those in thyroid tumours, which produce hormones) or neuroectodermal cells (secrete neurotransmitters like epinephrine in pheochromocytomas). Cells from those tumours are fragile, giving them a characteristic appearance. They exfoliate in several packets of cells with poorly defined cell margins and are surrounded by many bare nuclei, belonging to the ruptured cells. Because of this, they are sometimes called "naked nuclei" neoplasms (Raskin et al., 2023).

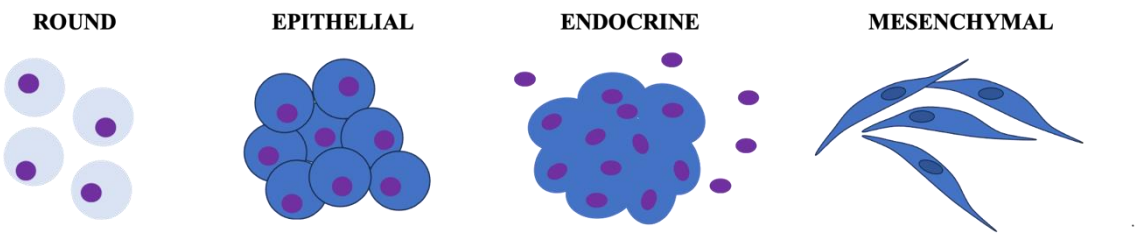


Figure 6: Schematic images of different cell tumour types based on cytological morphology and arrangement.

To help differentiate a hyperplastic process from a neoplastic lesion and to further classify the neoplasia into benign or malignant, cytological criteria of malignancy are used. It evaluates the presence of morphological abnormalities compared with the normal original cells. The criteria of malignancy are generally described by analysing the anisocytosis and the anisokaryosis. Anisocytosis refers to the size variation among cells from the same population, and anisokaryosis refers to the variation in the nuclear size. Other nuclear and cytological criteria are also evaluated (Cian and Monti, 2019; Raskin et al., 2023). See examples in Figures 7 A and 7 B.

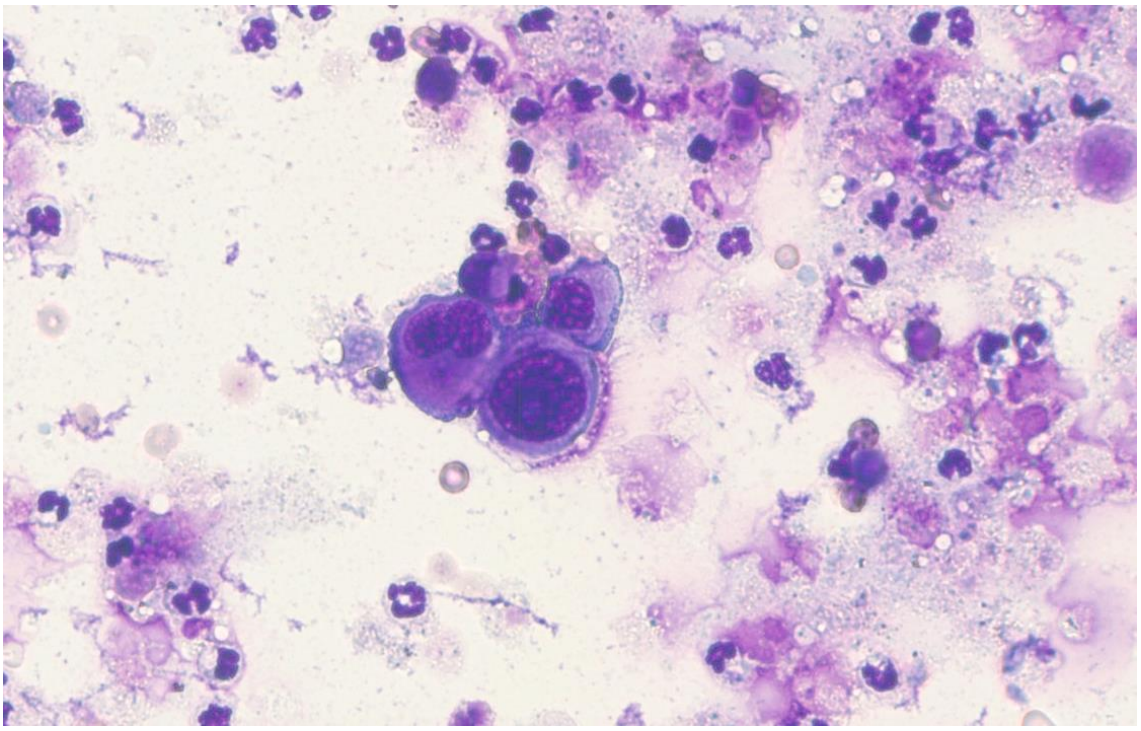


Figure 7 A: Dog. Prostatic carcinoma. Epithelial clusters showing marked anisocytosis and anisokaryosis, including binucleation, nuclear moulding and prominent macronucleoli.

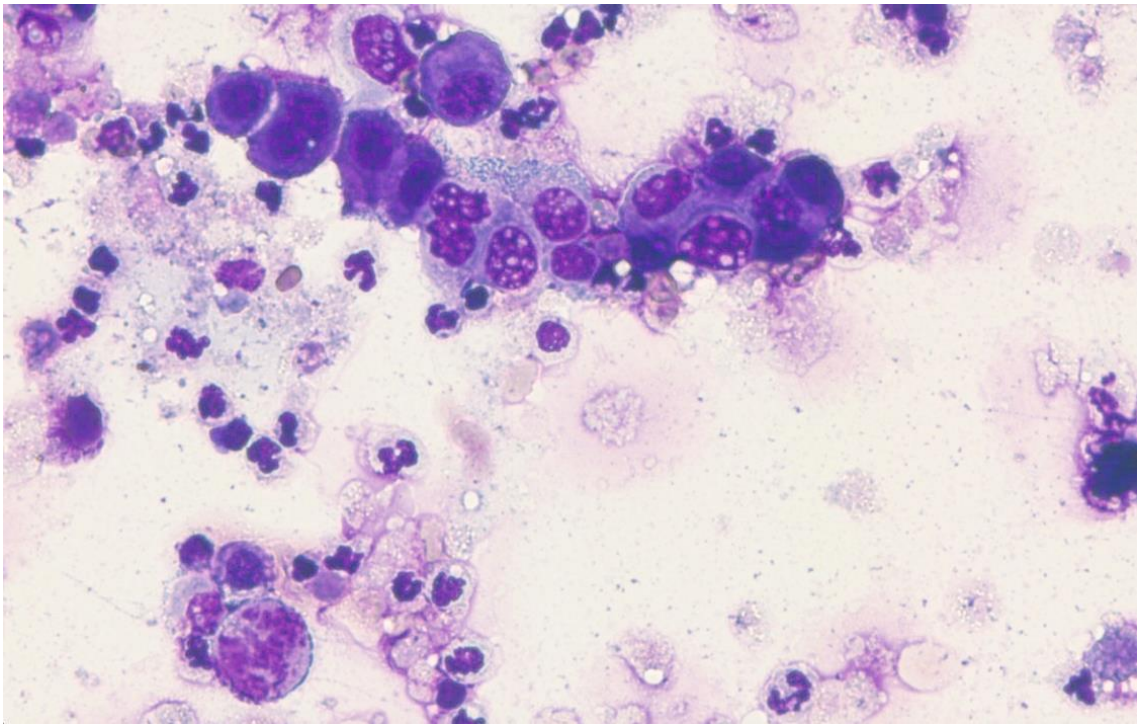


Figure 7 B: Dog (same case A). Prostatic carcinoma. Epithelial clusters showing marked anisocytosis and anisokaryosis with atypical mitotic figures (40x).

1.4.- Sampling techniques

Several different specimens are currently being evaluated by cytology in clinical practice, and different cytological collecting methods will be applied depending on the type of tissue or lesion. The most commonly used method is fine-needle aspiration (FNA). FNA helps assess solid tissues that include external tissues, like skin and subcutaneous tissues, and thoracic or abdominal organs, including internal reproductive organs like the ovaries. This technique consists of inserting the tip of the needle into the tissue of interest after appropriate cleaning and disinfecting of the cutaneous zone. The needle is advanced and retracted in different directions to allow the material to enter the needle. In small animals, this collection technique is generally achieved with a 22- or 25-gauge, 1- to $1^{1/2}$-inch needle firmly attached to a 6- or 12-cc syringe. However, in-depth organs like the ovaries, which are more difficult to reach, longer needles can be used (Raskin et al., 2023).

Although FNA can be performed with or without aspiration, the fine needle with aspiration is related to higher blood contamination, especially in vascular tissues like the liver, spleen, kidney and thyroid, among others. Regarding vascular tissue, fine needle biopsy without aspiration will be preferred (Raskin et al., 2023).

Aside from the type of tissue, the needle gauge can also affect the sample quality. A recent study comparing the needle gauge with the quality of the samples concluded that the samples taken with a 25-gauge needle contained

less blood contamination but increased cellular trauma. However, the ability to make a diagnosis was concluded not to be affected by the gauge size (Arai et al., 2019).

In FNA, the sample obtained is prepared by using the squash technique. This technique consists of placing a small amount of material on a clean glass slide approximately 1 cm from the frosted end. Then, a second one is placed over the specimen and is gently compressed between the two slides (Raskin et al., 2023).

Although FNA with squash is the most commonly used technique to obtain cytology specimens from solid tissues, other techniques may be used. Touch imprint is used for incisional or excisional biopsy samples. An incisional biopsy can be applied to a soft tissue or bone marrow core and consists of the removal of a representative sample of the tissue without removing it completely. An excisional biopsy consists of removing the complete lesion or tissue and can be applied to masses, lymph nodes, eyes, and testicles, among others. In both processes, specimens are obtained by the exfoliation of the cells when the tissue surface is touched to a glass slide (Raskin et al., 2023).

Scraping biopsy consists of scraping the surface of the skin with a scalpel blade or a razor to take cell samples from the superficial layers of the skin. This technique is mainly used in the skin, but it can also be applied to the conjunctiva or other firm tissues (Raskin et al., 2023).

In fluid samples like pericardial, abdominal or thoracic effusion, as well as for synovial fluid, urine or cerebrospinal fluid, samples are obtained by the aspiration of the fluid with a needle or a butterfly needle attached to a syringe. After the obtention of the sample, a direct and, if necessary, centrifuged smear is prepared. Direct smears can be prepared using the glass-slide blood film method. It consists of placing a drop of fluid at one end of a clean glass slide. Then, a second glass slide is positioned at a 30-degree angle to the first one, in front of the drop. This second slide is gently moved into the drop, and as soon as the fluid starts to spread along it, the slide is quickly pushed forward along the length of the first slide, distributing the sample in a shape resembling a flame (Harvey, 2012).

In liquid samples, the cytological interpretation should be combined with the cellularity and protein content. Furthermore, in bloody fluids, a buffy coat smear can be prepared. This technique consists of filling a microhematocrit capillary tube with the fluid, sealing one end with clay, and then centrifuging it for 4 to 5 minutes at 11000 to 12000 rpm. After the centrifugation, the bloody fluid is separated into three different layers based on density. Erythrocytes will be placed at the bottom, followed by a white layer, known as the "buffy coat," that will be placed between erythrocytes and plasma, which will be located at the top. The buffy coat contains white blood cells, larger cells, and other larger particles, such as parasites. To collect the buffy coat sample, the capillary tube is broken just above the buffy coat, and the clay is pushed with a needle until the buffy coat falls onto a glass slide. Then, the traditional squash technique is applied (Harvey, 2012).

The swab technique can be used to take specimens from the vagina, endometrium, oral cavity, ocular conjunctiva or faecal samples. Swabbings consist of rotating the swab against the tissue to allow the cells to be transferred. Then, the swab is rolled gently onto a clean glass slide. In the case of endometrial cytology, some authors recommend using a sterile speculum to facilitate the entrance into the endometrium as well as to reduce the incidence of sample contamination. (Raskin et al., 2023; Agámez et al., 2023).

Finally, washes (also named lavages) are also frequent cytological techniques to obtain samples from the prostate, urinary bladder, respiratory tract and endometrium, among others. Lavages consist of flushing cells from the superficial lining by introducing a small amount of fluid, generally phosphate-buffered saline (PBS), into the cavity and then recovering the material to obtain the sample. (Raskin et al., 2023).

1.5.- Cytology and the female reproductive tract

In the evaluation of female reproductive health, cytology is a commonly used tool in both human and veterinary medicine. Different special collection techniques can be used according to the site of sampling. For example, the cotton swab is a commonly used method for the obtention of vaginal samples for the staging of the oestrous cycle in species like dogs and cats. It consists in introducing a saline-moistened cotton swab craniodorsally into the caudal part of the vagina. Later, when the swab is located cranially to the urethral orifice, the swab is gently passed over the epithelial lining to obtain vaginal cells. Avoiding the vestibule and the clitoral fossa is crucial because the keratinised superficial cells in these sites can misinterpret the cytology. After collecting, the cotton swab is gently rolled into a clean glass microscope slide to transfer the cells. The next step is the staining procedure. Although different types of stains have been used for vaginal preparations, the most commonly used are methanolic or aqueous Romanowsky stains (Raskin et al., 2023).

Fine needle aspiration or cytological imprints are also helpful to detect vaginal inflammation and neoplasia (Raskin et al., 2023) (Figures 8: A and B).

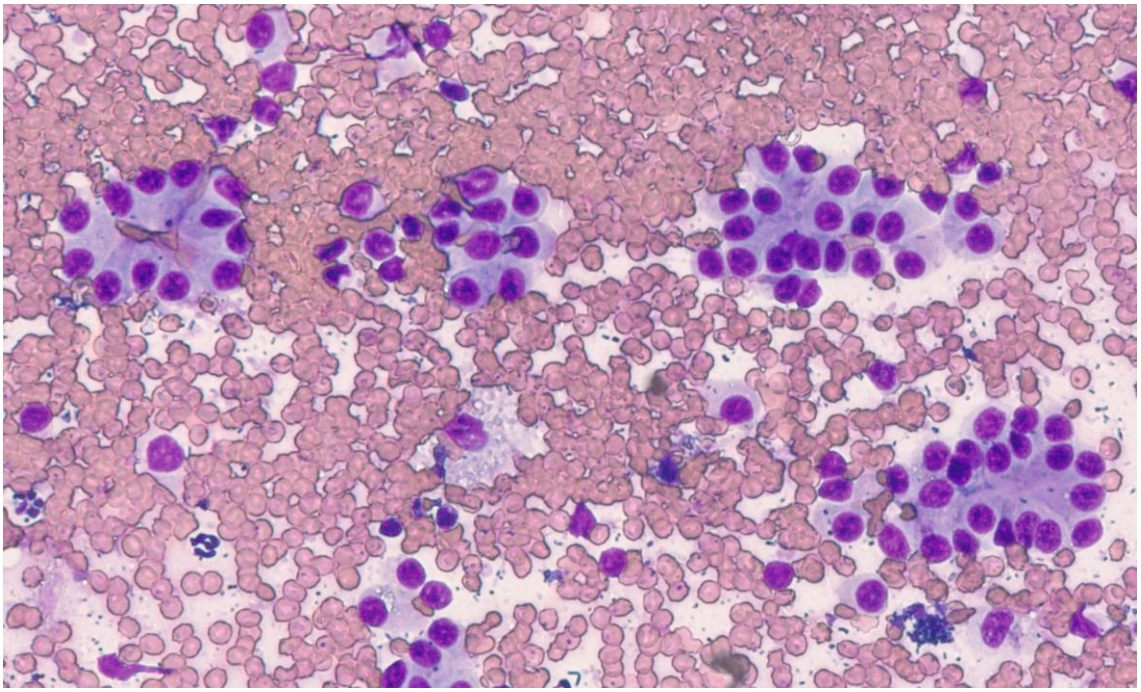


Figure 8 A: Dog female vaginal mass. A: FNA from the mass. Cytology is highly suggestive of the presence of a clitorial adenocarcinoma (40x).

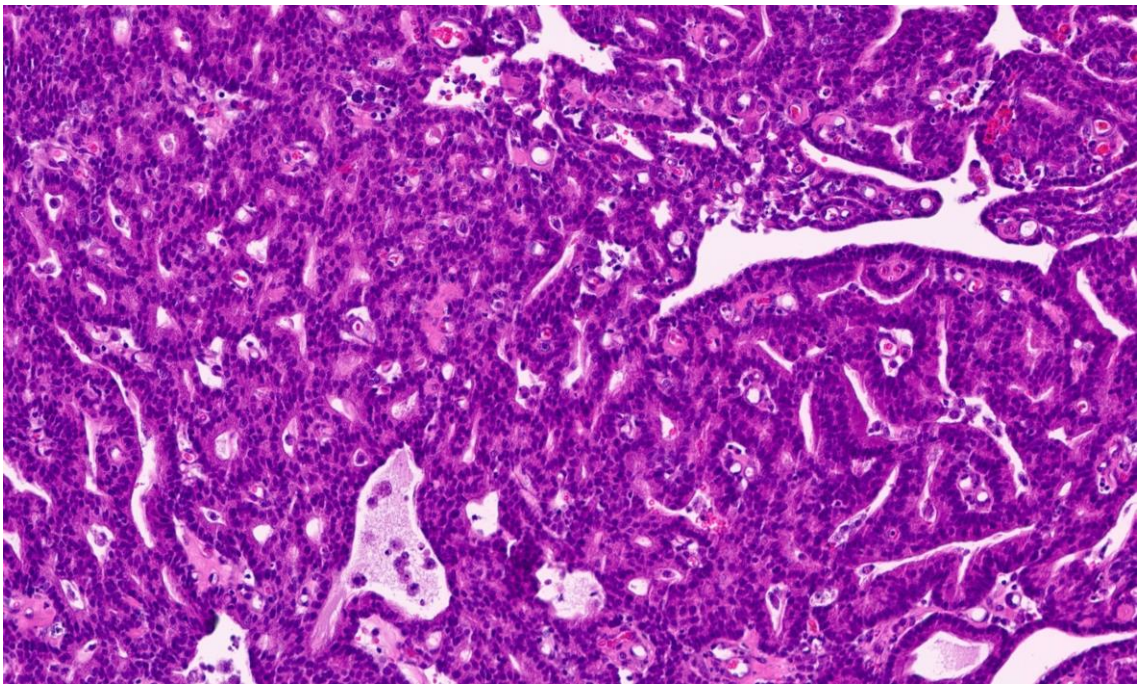


Figure 8 B (same case): Histological section from the excised mass indicates an epithelial glandular neoplasm and supports a clitoral adenocarcinoma.

Uterine cytology in dogs and cats is indicated to evaluate the fertility status and different pathologies such as cystic endometrial hyperplasia, inflammatory disease and neoplastic processes. Samples can be obtained at the time of hysterectomy or transcervically using an endoscope to visualise the cervix and passing a catheter through

the cervix into the uterus. Samples are generally obtained by the infusion and aspiration of sterile normal saline (Raskin et al., 2023).

In large species like cows or mares, endometrial cytology is also broadly used, and it has been demonstrated to increase the capacity to detect endometritis in subfertile animals, especially when used in combination with endometrial culture and/or biopsy (Baranski et al., 2012; Nielsen, 2005) (Figure 9). Uterine culture swabs, double-guarded culture swabs, uterine cytology brushes, uterine biopsies and specimens resulting from uterine lavage fluids have been reported for the collection of endometrial cytology specimens. Both swabs and brushes have a similar technique. The veterinarian must wear sterile sleeves and place the tip of the device into the palm. Then, the hand with the device is passed through the vulva, vestibule, and vagina until the cervix is reached. It is important to avoid contact with the lubricant, the skin or the mucosa whilst passing through the vulva and the vagina. Once in the cervix, a finger guides the device until the uterine lumen is reached. Rolling the device against the endometrium for at least 30 seconds is recommended. After that, the device is retracted to obtain the sample. Although swabs and brushes are both applied similarly, some practitioners prefer brushes. Cytological brushes achieve a higher proportion of diagnostic smears, with a higher proportion of well-preserved cells and fewer artefacts (Cocchia et al., 2012; Agámez et al., 2023). In mares, the use of a vaginal speculum to obtain endometrial cytology is less common than in dogs and cats. However, it is advantageous as it reduces the incidence of sample contamination, especially for microbiological specimens (Agámez et al., 2023).

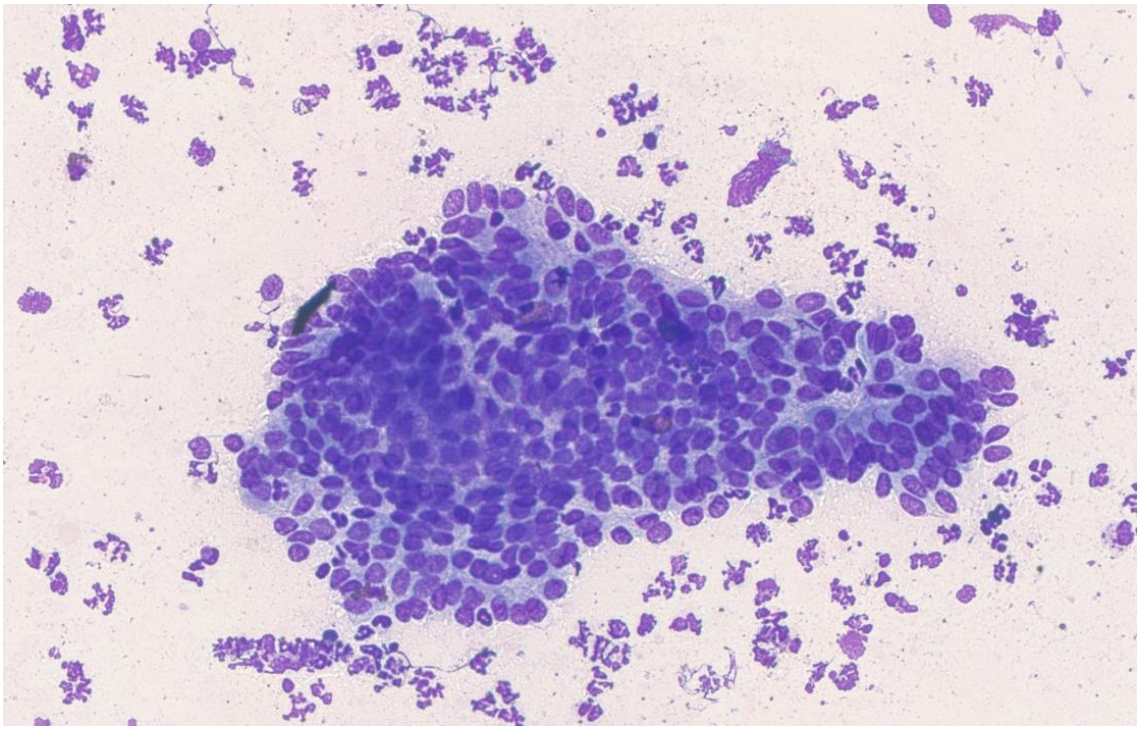


Figure 9: Horse. Uterine cytology in a mare with infertility. Abundant numbers of neutrophils are detected and some are admixed with endometrial cells, indicating endometritis (30x).

In bitches, uterine flushing with transcervical uterine cannulation has been described as a technique to obtain uterine cytology. The study of the morphology of endometrial cells coupled with morphometric features was proposed as a tool to distinguish uterine disorders and support a diagnosis (Groppetti et al., 2010; Praderio et al., 2019).

Ovarian fine-needle cytology is commonly used in human medicine and has been demonstrated to be a good diagnostic tool for ovarian tumours in dogs (Bertazzolo et al., 2004). Furthermore, some authors have described the main specific cytological characteristics of the normal canine ovaries in different oestrous stages to help identify pathological states affecting this organ (Piseddu et al., 2012). In bitches, cytology demonstrates a very good agreement (94,7%) with histopathology to recognise ovarian neoplastic lesions. Samples can be obtained at the time of ovariohysterectomy or by ultrasound-guided percutaneous FNA (Bertazzolo et al., 2004).

Finally, mammary lesions can also be evaluated by cytology. Samples are commonly obtained by fine-needle aspiration. However, imprints and excretions from the gland can also be used. When a tumour is suspected, it is recommended to aspirate in different locations to avoid misdiagnosis due to tissue heterogeneity (Raskin et al., 2023).

1.6.- Infertility: a worldwide problem

Infertility is defined as the failure to conceive after a period of unprotected and regular sexual intercourse. In human medicine, infertility is considered a worldwide, highly prevalent problem that affects around 8% to 12% of couples of reproductive age (Inhorn & Patrizio, 2015; Ombelet et al., 2008). This percentage has higher ranges in countries with poor access to assisted reproductive techniques (Mascarenhas et al., 2012).

According to the World Health Organisation (WHO) clinical definition, infertility in a woman is considered when, within 12 months of unprotected intercourse or therapeutic insemination, a pregnancy has not been achieved. Two types of infertility are defined in human medicine: primary infertility and secondary infertility. Primary infertility is the inability to conceive one child for the first time, while secondary infertility is the failure to conceive a child following a prior pregnancy. Secondary infertility is frequently associated with reproductive tract infections, and it is more prevalent in regions with poor maternity care or high rates of abortions (Inhorn & Patrizio, 2015). Although historically, the female factor has been more frequently associated with infertility, it has been demonstrated that the male factor plays a role in 50% of infertile couples and is the only contributor to infertility in 20% of cases (Chu et al., 2019).

In women, the principal causes of infertility are classified as ovulatory dysfunction, tubular occlusion, endometriosis, diminished ovarian reserve and uterine and cervical factors. Among them, ovulatory diseases

and tubal diseases are the most frequently related to infertility. However, lifestyle and environmental factors like obesity, stress or smoking also have a relevant impact (Carson & Kallen, 2021).

1.7.- Infertility in Veterinary Medicine

In veterinary medicine, infertility is also a medical problem with a high economic impact. Fertility problems are well-studied in large animals like cows and mares, but not so much in companion animals. In that species, several causes may impact fertility. Endometritis is one of the leading causes and is defined as the inflammation of the uterine mucosa that extends no deeper than the stratum spongiosum and is commonly secondary to an infectious aetiology (Pradeiro et al., 2019).

Endometritis has been widely studied in mares and cows. The uterus contains commensal organisms (bacteria, viruses and yeast/fungi). Endometritis occurs when a shift in the numbers or the type of organisms, together with an immune dysfunction, develops uterine infection and inflammation. In mares, it has been related to being breeding-induced or secondary to exposure to semen (Pascottini et al., 2023; Ravaioli et al., 2022; Troedsson, 2006). In dairy cows, postpartum endometritis is considered the principal cause of infertility. Uterine contamination at parturition or during the following days occurs in 80-100% of the animals, and it is related to bacterial presence in the uterine lumen. Most cows successfully deal with bacterial contamination, but approximately 15-20% of the animals develop endometritis (Walsh et al., 2011). In those high-producing animals, the reduction of immune competence is common and is associated with a higher risk of endometritis, mastitis and lameness. Both mastitis and lameness have been related to higher numbers of services to achieve conception in comparison with healthy cows (Walsh et al., 2011).

Sustained endometritis may lead to the appearance of endometriosis. This condition is a degenerative and chronic process that is characterized by the development of fibrosis in the endometrium. Endometriosis is one of the major causes of subfertility/infertility in mares (Rebordao et al., 2014).

Environmental management factors such as age, nutrition, training, temperature at mating, and breeding season, among others, also play an important role in infertility in mares and cows, as in other animals (Laseca et al., 2014; Walsh et al., 2011). In cattle, reduced fertility is mainly described in the summer period and is recognised to be associated with several factors such as temperature, humidity, photoperiod and food intake (De Rensis et al., 2017).

During the breeding season, different factors can affect the fertility of cows. Failure to detect the oestrus and the reduced duration of detectable oestrus play a crucial role in reduced fertility rates. Furthermore, during the fertilisation period, heat stress, oocyte quality, and sperm characteristics are some of the reasons for fertilisation

failure. Nonetheless important, embryo mortality is also a major concern and is frequently associated with prior uterine infection and the development of small embryos due to inadequate uterine environment secondary to low progesterone concentrations (Walsh et al., 2011).

Genetic factors are another cause that may impact reproductive performance. Furthermore, some reproductive traits may be more affected by environmental factors. In that field, the current exponential expansion in horse genomics can help detect which genotypes may be affected by environmental factors and also detect the physiological functions that can affect a specific genotype (Laseca et al., 2021). Today, the use of genomics in mares is still limited in contrast with other livestock animals like cattle or pigs, where those methods are largely developed (Laseca et al., 2021).

In small animals, infertility and subfertility are also common problems. In the bitch, infertility is most commonly associated with inappropriate breeding management rather than a true inability to conceive (Grundy et al., 2002). However, pathologies associated with infertility also exist. Common problems affecting the endometrium are endometritis and cystic endometrial hyperplasia. Both of them are related to reduced fertility, and those changes frequently occur together. Endometritis in the bitch is commonly associated with bacterial infection (Fontaine et al., 2009; Gifford et al., 2014). Other less common clinically relevant endometrial lesions that can reduce fertility are uterine fibrosis and eosinophilic endometritis (Gifford et al., 2014).

There are also other pathologies not related to the endometrium that can impact fertility and include oocyte incompetence, ovulatory failure, oviductal defects, and endocrinopathies, among others (Gifford et al., 2014).

1.8.- Infertility in cats

Much less is known about infertility in queens compared with other domestic animals. However, recent reports describe that infertility in cats can occur in at least 20% of some pedigree cats. This represents an important problem for breeders as it causes economic and genetic losses (Fontbonne et al., 2020; Niewiadomska et al., 2023). Recent studies performed in breeding colonies have demonstrated that mature breeding queens can start with fertility problems as early as 3 years of age. In these animals, reduced fertility was considered after infertile matings or reduced litter size, and the most common causes related were undiagnosed cystic endometrial hyperplasia, endometritis, pyometra and ovarian cysts (Johnson et al., 2023).

As with the other species described, there are many different causes of infertility in queens and are generally categorised as failures during mating (including both time of mating or problems during the intercourse), uterine diseases, infectious processes, endocrine pathologies, chromosomal or genetical abnormalities, inadequate nutrition and anestrous (Fontbonne et al., 2020).

The inappropriate mating time is often a frequent cause of infertility. It has been demonstrated that follicular oocytes ovulated after the first day of oestrus may be immature or of poor quality. It is recommended to leave the queens with the males at least after three matings, as there is a high percentage of queens that ovulate after multiple copulations (Fontbonne et al., 2020).

Spontaneous ovulation is another problem in catteries and may occur despite having an appropriate mating time and conditions. This phenomenon occurs when ovulation appears without a coitus. It is more frequent in breeding environments where numerous females live together. It is a real problem as the female has already ovulated when it is presented to the male for mating (Fontbonne et al., 2020).

Anovulatory cycles may be related to a low number of matings or insufficient ovulation stimulation. Furthermore, stress and inexperience may also play an important role in infertility. Some authors also point out that there are breeds like Persian or Maine Coon that have low-libido males (Fontbonne et al., 2020).

Although the impact of each of the causes affecting fertility has not been well established, it has been hypothesised that uterine problems could be one of the major causes of infertility in the queen (Fontbonne et al., 2020; Niewiadomska et al., 2023). Endometritis may also play an important role in infertility, especially low-grade endometritis, which is very difficult to diagnose, and its incidence and importance as a cause of infertility are still unknown (Fontbonne et al., 2020).

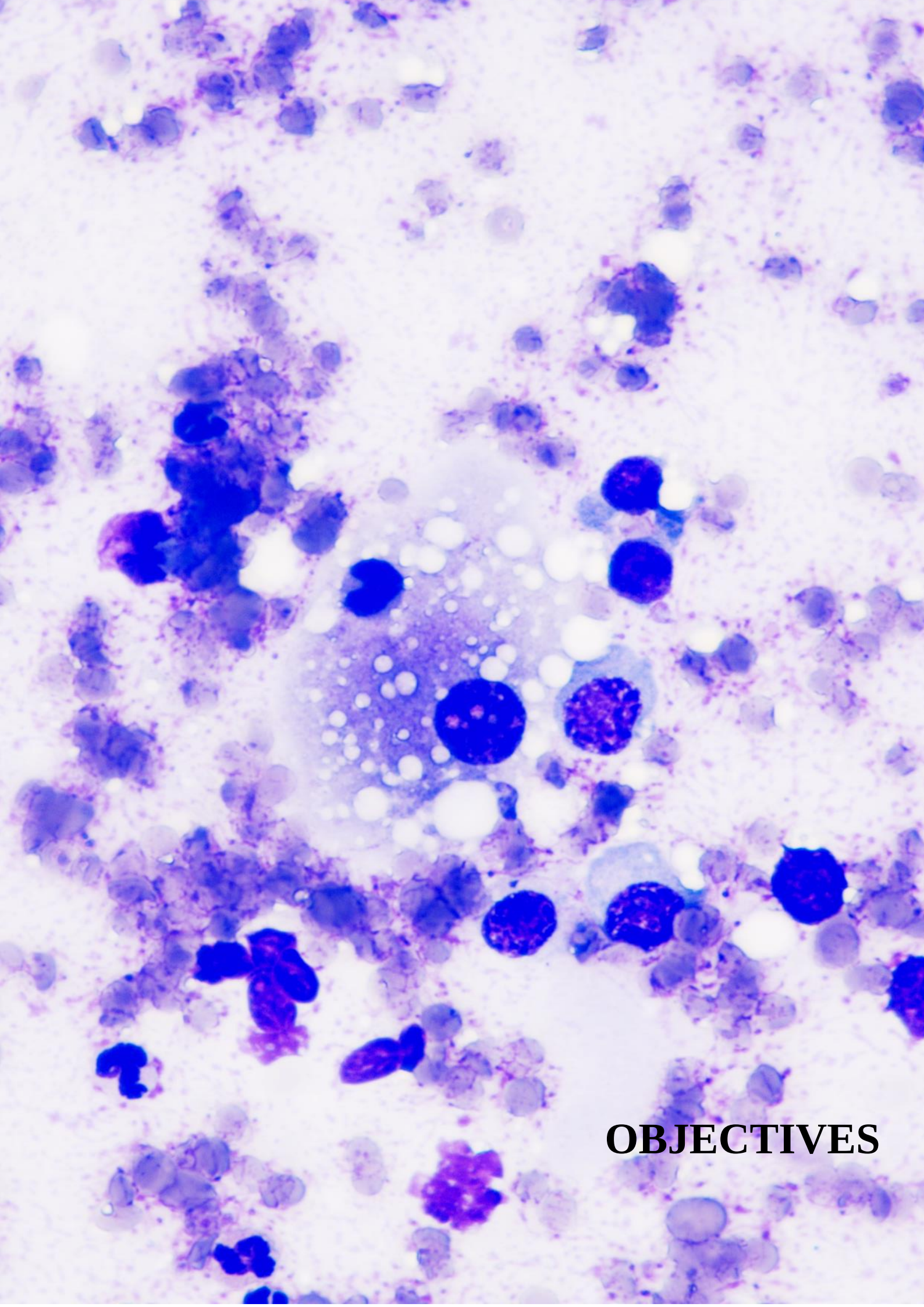
On the other hand, in a recent case series, endometrial hyperplasia was detected to be the most prevalent endometrial pathology, and it was associated with the inability of embryo implantation and adequate placental development (Niewiadomska et al., 2023). Cystic endometrial hyperplasia is more common in old queens or queens that have presented several episodes of spontaneous ovulation. Furthermore, it may also occur after the use of progestins. Finally, hydrometra and mucometra may also be associated with infertility in young queens, but the exact cause is often impossible to determine (Fontbonne et al., 2020).

Infectious agents, especially some viruses (FeLV, FIV, Coronavirus) and bacteria (*Chlamydophila felis*), have an important impact on fertility by preventing implantation and/or embryonic development and inducing pregnancy loss (Fontbonne et al., 2020).

Endocrine diseases like hyperoestrogenism can also impair reproduction. Hyperoestrogenism may occur secondary to granulosa cell tumours or follicular cysts. The lack of progesterone to maintain the pregnancy has also been suspected in some queens, and Maine Coons seem to be more affected than other breeds (Fontbonne et al., 2020).

Finally, nutrition deficiencies, chromosomal and genetic problems, and miscellaneous conditions such as environmental pollutants are less frequently related to infertility but could also play an important role (Fontbonne et al., 2020).

In contrast with large animals, the reproductive tract inaccessibility in dogs and cats makes it challenging for the clinician to obtain proper diagnostic information. In these species, samples for uterine cytology and biopsy can be obtained, but these procedures are more invasive than in other species and require a higher level of expertise (Wilborn & Maxwell, 2012).

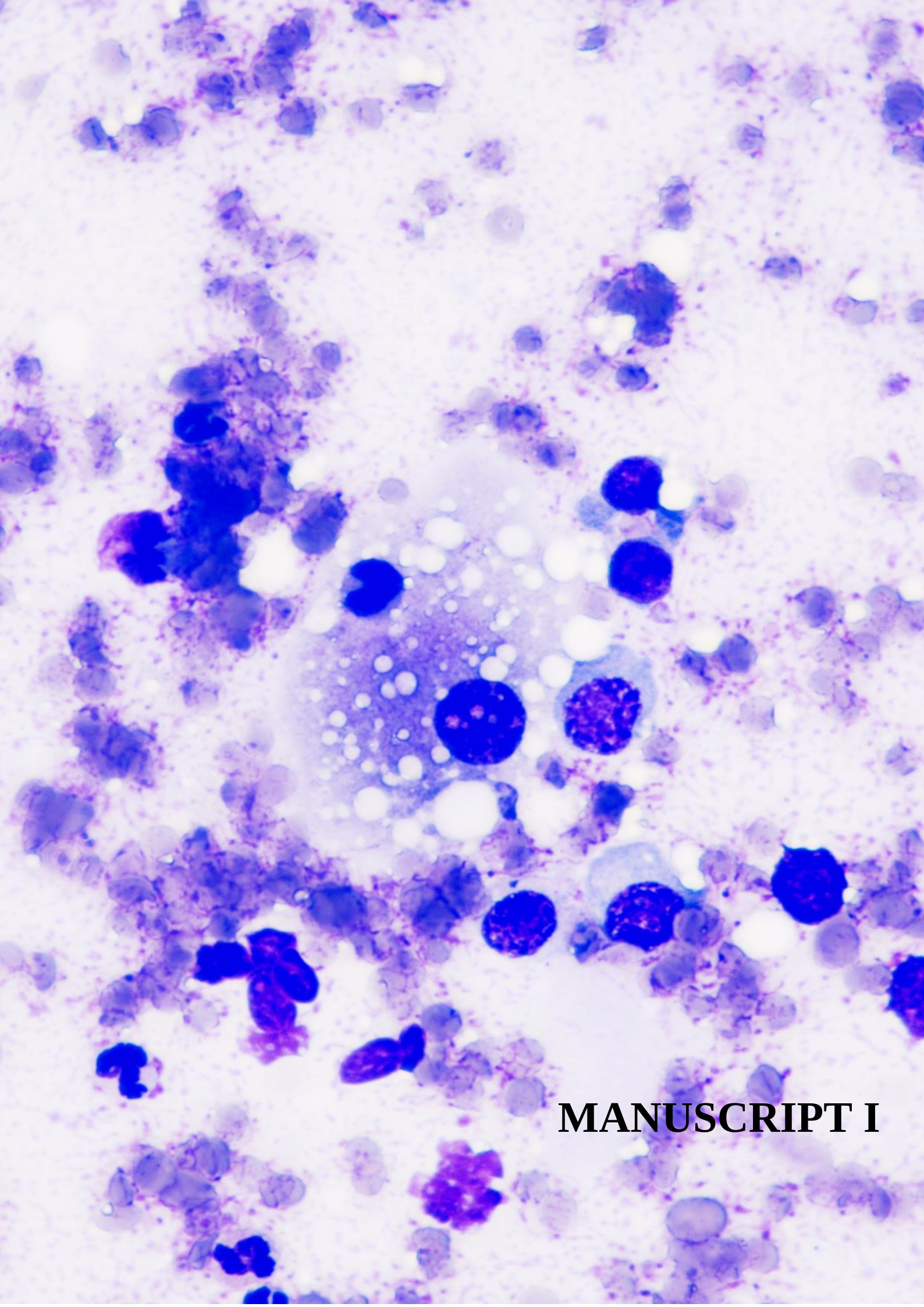


OBJECTIVES

VI. OBJECTIVES

The main objectives proposed for this PhD thesis were:

1- Evaluating the usefulness of cytology for the assessment of the queen's reproductive tract, specifically, the uterus and the ovaries.
2- Describe the cytological characteristics of the uterus and the ovaries in healthy queens.
3- Determine if the cytology can be used as a diagnostic technique for a prompt diagnosis of endometritis in queens.



MANUSCRIPT I

VII. MANUSCRIPT I

Endometrial Status in Queens Evaluated by Histopathology Findings and two Cytological Techniques: Low-Volume Uterine Lavage and Uterine Swabbing

Alba Martí, Anna Serrano, Josep Pastor, Teresa Rigau, Ugné Petkevičiut, Maria Àngels Calvo, Esteban Leonardo Arosemena, Aida Yuste, David Prandi, Adrià Aguilar and Maria Montserrat Rivera del Alamo

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Endometrial Status in Queens Evaluated by Histopathology Findings and Two Cytological Techniques: Low-Volume Uterine Lavage and Uterine Swabbing

Alba Martí [1], Anna Serrano [1], Josep Pastor [1,*], Teresa Rigau [1], Ugné Petkevičiuté [1], Maria Àngels Calvo [2], Esteban Leonardo Arosemena [2], Aida Yuste [2], David Prandi [1], Adrià Aguilar [1] and Maria Montserrat Rivera del Alamo [1]

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Simple Summary: The endometrium health of feline queens can be difficult to assess due to the reduced size of the uterus, which hinders representative biopsy sampling. This may result in limitations in diagnosing endometritis, and consequently in detecting infertility problems. Although histology is considered the most reliable technique for diagnosing endometritis in many species, cytology is also gaining importance and may be an alternative tool for evaluating the endometrium in small species. Two different common cytological techniques (uterine lavage and uterine swabbing) were compared to determine the reliability of cytology for evaluating the endometrium status in queens. Histopathological and bacteriological information was used for the control methods. Our results demonstrated that cytology may be a useful diagnostic tool for assessing the endometrial status. In addition, when comparing cytological techniques, the uterine lavage method was more representative than uterine swabbing.

Abstract: Endometritis is associated with fertility problems in many species, with endometrial biopsy being the main diagnostic tool. In feline queens, the reduced size of the uterus may make it difficult to obtain representative diagnostic samples. Endometrial cytology may represent a valuable diagnostic tool for evaluating the health status of the endometrium in queens. Fifty domestic shorthair queens were included and divided into two cytological diagnostic technique groups, the uterine lavage (UL; n = 28) and uterine swabbing (US; n = 22) groups. Cytological results were compared with histopathological and bacteriological information. Changes in the histopathological patterns were also evaluated and compared with progesterone levels to confirm previous published data. Furthermore, the results from both cytological sampling methods were compared to evaluate the utility of each method. Endometritis was ruled out in all queens by means of histology and microbiology. Leukocyte counts and red blood cell/endometrial cell ratios were significantly higher in US than UL samples. Additionally, UL sampling is less affected by blood contamination and cells are better preserved. The combination of endometrial cytology and uterine culture might be useful for evaluating the endometrial characteristics in queens. The UL evaluation method is more representative of the actual endometrial status than the US technique.

Keywords: uterus; cytology; polymorphonuclear neutrophil; queen

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1. Introduction

Endometritis is defined as inflammation of the uterine mucosa not extending deeper than the stratum spongiosum [1]. It has been widely associated with infertility, recurrent implantation failure, impaired embryo survival, and pregnancy loss in many species, such as mares [2,3], cows [4,5], bitches [6], and women [7].

However, endometritis is not always associated with evident clinical signs, with infertility being the only detected alteration [6,8,9]. In fact, recent publications on bitches support the notion that subclinical endometritis is a frequent finding in clinically healthy animals [6]. In cows, this condition is well defined and animals that undergo endometritis without evidence of any other clinical sign are also recognized as having subclinical endometritis, also named cytological endometritis [5,10]. In subclinical endometritis, endometrial cytology is characterized by the presence of polymorphonuclear neutrophils (PMN) with absent or minimal intrauterine exudate [5,11,12]. In human medicine, asymptomatic endometritis is known as chronic endometritis, and it is also associated with recognized fertility problems. It has a specific histological pattern that is characterized by the presence of a subtle lymphoplasmacytic inflammation of the endometrium [7]. In queens, studies on endometritis are scarce and previous authors agree that subclinical endometritis is difficult to evaluate [13,14], hampering elucidation of the actual prevalence in this species. The exact etiology of subclinical and chronic endometritis in healthy females has not been completely understood and seems to be different depending on the species [6]. In most species, endometritis is diagnosed by means of cytological and bacterio- logical evaluation of endometrial samples, in combination with ultrasonographic and histopathological assessment [15–17], being the latest and most reliable tools used to diagnose endometritis in many species, such as mares [18,19], bitches [20], and cows [21]. In queens, the reduced size of the uterus may make it difficult to obtain representative tissue samples for histopathological examination. This may limit the diagnosis of possible endometritis. Thus, finding an easy, safe, and reliable technique other than endometrial biopsy to evaluate the endometrium in queens would open the possibility for the diagnosis of endometritis in this species. Furthermore, endometrial cytology is gaining importance as a diagnostic technique. In cows, some authors consider endometrial cytology as a potential reference test because it can detect both clinical and subclinical endometritis [11]. In other species such as mares [9] and women [22], endometrial cytology is also considered a valuable tool for the diagnosis of certain uterine diseases. No information exists in cats using this diagnostic technique.

The main aim of this study was to determine which cytological sampling method, either (1) low-volume uterine lavage (UL) or (2) uterine swabbing (US), is more reliable for evaluating endometrial status in queens. Histological and microbiological analyses were used as control techniques to confirm the actual endometrial status.

2. Materials and Methods

2.1. Animals

A total of 50 domestic shorthair queens of different ages were included in this study. Queens belonged to a neutering program of stray cats carried out by the Surgery Unit of the Facultat de Veterinària of the Universitat Autònoma de Barcelona (UAB). No information about the previous reproductive history, age, or reproductive status of the queens was available. Before the inclusion, a complete physical examination and tests against feline leukemia virus and feline immunodeficiency virus (Snap FIV/FeLV; Idexx Laboratories, Westbrook, ME, USA) were performed. Only those animals that were healthy and negative against both diseases were included in the study.

Prior to the surgery, queens were pre-medicated with an intramuscular combination of 5 mg/kg ketamine (Ketamidor®, Richer pharma) with 20 µg/kg buprenorphine (Buprecare®, Divisa FramaVic, S.A) and 0.2 mg/kg midazolam (Midazolam®, Normon S.A). When each animal reached sedation status, a blood sample from the jugular vein was

collected and placed into a glass tube. The blood was allowed to clot and then centrifuged at 2200× *g* for 10 min at room temperature. The serum was immediately frozen and kept at −20 °C until analyses were performed.

After blood collection, queens were induced intravenously with 2–4 mg/kg propofol (Propovet TM, Zoetis Ecuphar). Anesthetic status was maintained using 1.5–2% isoflurane (IsoFlo, Zoetis Ecuphar) in oxygen using a Mapleson F anesthetic breathing circuit. When the anesthetic status was reached, a conventional ovariohysterectomy was performed via ventral midline laparotomy.

After ovariohysterectomy was performed, uteri were randomly divided into two groups according to the technique used to obtain the cytological preparations, namely the uterine lavage group and uterine swabbing group. The uterine lavage group (UL) included a total of 28 queens, while the uterine swabbing group (US) included a total of 22 queens.

The experimental procedure was approved by the Ethical Committee for Animal Care and Research of the UAB (CEEAH, code 2939).

2.2. Sample Collection

In the UL group, a total of 2 mL of phosphate-buffered saline (PBS) was injected into the uterine lumen through the uterine wall using a 2 mL sterile syringe with a 23G needle. The solution was instilled and at least 0.5 mL was aspirated back. The first drop was discarded and the next 2–3 drops were used for microbiological purposes. A sterile swab (Eurotubo®, invasive sterile collection swab, Deltalab; Rubí, Spain) was soaked and then introduced into a transport medium tube. The remaining volume was then placed in a 2 mL Eppendorf tube for the cytological evaluation, which was processed within 1 h of collection. A 50 µL aliquot was centrifuged at 3330× *g* for 5 min (Rotofix 32A, Andreas Hettich GmbH and Co., Tuttlingen, Germany) and a smear from the pellet was performed. In the US group, a longitudinal incision with a scalpel blade (Swann-Morton®, sterile carbon steel surgical blades; Sheffield, England) in the uterine horn was performed to gain access to the uterine lumen. A sterile swab (Eurotubo®, invasive sterile collection swab, Deltalab; Rubí, Spain) was then introduced through the incision and rotated to collect a sample from the endometrium surface. To preserve the sample, the swab was introduced into a transport medium tube and submitted for microbiological exam. After this, the incision was longitudinally elongated and another sterile swab was introduced and rotated to collect another sample. The sample was immediately smeared on a sterile glass slide to obtain the cytological sample.

Once samples for cytology and microbiology were obtained, a full-thickness biopsy for histopathological study was obtained and immediately placed in 10% paraformaldehyde.

2.3. Progesterone

Serum progesterone (P_4) was measured in order to establish the ovulation status of the queens. Those queens with progesterone concentrations below 1.5 ng/mL were considered non-ovulated, while those showing progesterone levels above 1.5 ng/mL were considered ovulated [23–26]. Serum progesterone concentration was determined using an Inmulite 1000 instrument (Immulite; Siemens Healthcare Diagnostics, Cornellà del Llobregat, Barcelona, Spain). The intra-assay and inter-assay coefficients of variation (CV %) were 5.5 and 6.5, respectively.

2.4. Microbiology

Microbiological evaluation was performed following the protocols established by the Applied and Environmental Microbiology Laboratory of Animal Health and Anatomy Department of the Veterinary Faculty (UAB). Samples were grown in blood agar (Columbia Agar supplemented with sheep blood), Man–Rogosa–Sharpe agar (MRS, tryptic soy agar (TSA), McConkey agar (MK), Baird–Parker agar (BP) supplemented with egg yolk tellurite emulsion, Sabouraud agar supplemented with 0.5 gr/L chloramphenicol, and tryptone sulfite neomycin (TSN) agar. All culture media were purchased from Liofilchem Srl (Italy), with the exception of Columbia agar, which was purchased from Bio-Rad Laboratories

(USA). Oxygen and temperature conditions for the different culture media are shown in Table 1.

Table 1. Oxygen and temperature conditions for the different culture media.

Agar Media	Oxygen Conditions	Temperature
Blood	Aerobic Anaerobic 5% CO_2	37 °C 37 °C 37 °C
MRS	Aerobic Anaerobic 5% CO_2	37 °C 37 °C 37 °C
TSA	Aerobic	37 °C
McConkey	Aerobic	37 °C
Baird–Parker	Aerobic	37 °C
Sabouraud	Aerobic	28 °C
TSN	Anaerobic	42 °C

MRS: Man-Rogosa-Sharpe agar; TSA: tryptic soy agar; TSN: Sabouraud agar supplemented with 0.5 gr/L chloramphenicol and tryptone sulfite neomycin agar.

Samples were processed within one hour of sampling. Swabs were streaked on Petri dishes following a continuous streak method. Then, plates were incubated for 24 h at the different stated conditions and an initial counting of colonies was performed. Those plates with negative growth were incubated during 24 more hours and the counting was repeated at the end of this second period of incubation. Plates in anaerobic conditions were incubated for 48 h with an Anaerocult system (Merck KGaA, 64271 Darmstadt, Germany), whereas Sabouraud agar plates were incubated for 7 days before being discarded.

2.5. Histopathology

Here, 4-µm-thick sections were obtained for histological examination. Samples were then stained with hematoxylin and eosin and evaluated according to previously reported data [27] through the NPD View 2 viewing software (Hamamatsu Photonics, Korea). The biopsy assessment included evaluation of the glandular density and diameter, height of the luminal epithelia, and white blood cell counts.

Glandular density was evaluated by counting the number of glands in 10 randomly selected fields at 400× magnification in both the stratum spongiosum and the stratum compactum. Glandular diameter was evaluated by considering the mean of two perpendicular diameters of each gland (from the basal lamina to the opposite one), measured in the stratum spongiosum. A total of 30 randomly selected glands were studied at 400× magnification. The height of the luminal epithelia was determined by measuring a total of 30 randomly selected cells from the basal lamina to the apical membrane, recorded at 400× magnification. Neutrophil counting was performed by counting in 10 randomly selected fields at 400× magnification. A mean for each variable was calculated for each sample.

2.6. Cytology Samples

Both UL and US samples were stained using an automatic dye (Hematek®, Siemens Healthcare Diagnostics INC, Tarrytown, NY, USA) with a modified Giemsa stain (Auto-Hemacolor®, Merck KGaAQ, Darmstadt, Germany), then examined using a light mi- croscope at ×100 to ×1000 magnification (Nikon Eclipse Ci, Nikon, Japan). Slides were examined separately for cytological analysis by a board-certified pathologist and a resident of clinical pathology. Endometrial cells, erythrocytes, and leukocytes were examined. The analyzed characteristics and classification scores are summarized in Table 2. The parame- ters studied from each sample were the smear quality, the smear cellularity (including the characterization of the endometrial cells), the calculated cells scores, and the evaluation of

inflammation focusing on the presence and disposition of polymorphonuclear neutrophils (PMN), as described below. For each smear, the mean of both observed results was used. When a discrepancy existed, a consensual result was obtained.

Table 2. Cytologic features evaluated in the smears obtained with the uterine lavage and the uterine swabbing sampling methods.

Cytological Feature	Score				
	0	**1**	**2**	**3**	**4**
Mucus or Proteinaceous debris	Absent/clear background	Present but adequate for diagnosis	Abundant	Excessive, not adequate for diagnosis	-
Blood in Background (RBC/40×)	Absent (0–5)	Scattered (6–50)	Few (51–150)	Moderate (151–300)	Abundant (>300)
Cellularity	Absent	Scattered	Moderate	Abundant	-
Cell Preservation (% of broken cells)	Adequate (0)	Good (<25)	Fairly adequate (25–50)	Not useful for diagnosis (>50)	-
Epithelial Cells (number of cells)	Absent (0)	Scant (1–40)	Few (40–80)	Moderate (80–199)	Abundant (>200)
Individual Epithelial Cells	Absent	Scant	Few	Moderate	Abundant
Clusters Epithelial Cells (number of clusters)	Absent (0)	Scant (<10)	Few (10–40)	Moderate (40–80)	Abundant (>80)
Acinar Arrangement (HPF)	Absent (0)	Scattered <1/10 HPF	Moderate 1–3/10 HPF	Abundant >3/10 HPF	
Shape	Round	Low Columnar	Columnar		
Pyknotic (%)	Absent (0)	Scant (<10)	Few (10–20)	Moderate (20–50)	Abundant (>50)
Cilia	Absent (0)	Scattered <1/10 HPF	Moderate 1–3/HPF	Abundant >3/10 HPF	
Vacuoles (%)	Absent (0)	Scattered (<20)	Moderate (20–60) Cells	Abundant (>60) Cells	
Nucleoli (%)	Absent (0)	Scattered <20	Moderate 20–60	Abundant >60	
Mitosis (Number of mitoses)	Absent (0)	Scattered 0–1/10 HPF	Moderate 1–3/10 HPF	Abundant >3/10 HPF	

HPF: high power field. RBC: red blood cells.

2.6.1. Smear Quality

The smear quality was assessed by evaluating the background, the blood dilution of the sample, and the presence of endometrial cells. The background was graded with a 0 to 3 score according to the presence of mucus or proteinaceous debris. A 0 score was given to smears without mucus or debris, a score of 1 was applied for those with the presence of mucus or proteinaceous debris that were adequate for diagnosis, a score of 2 was given to smears with abundant mucus or proteinaceous debris that were still adequate for diagnosis, and finally a score of 3 was given to samples with excessive presence of mucus and proteinaceous debris that were not adequate for diagnosis.

The erythrocyte content or blood dilution of the sample was evaluated using a similar approach as previously described [28]. A numerical score ranging from 0 to 4 was applied according to the number and distribution of red blood cells. A score of 0 was obtained when none or very few erythrocytes (0–5 RBCs/40× magnification) were present in the preparation. Smears scored as 1 were those with few dispersed erythrocytes (6–50 RBCs/40×). Samples scored as 2 showed moderate numbers of red blood cells, either dispersed or in

small clumps (51–150 RBCs/40×). Those with a score of 3 showed increased numbers of erythrocytes frequently organized in different sized clumps (151–300 RBCs/40×). Finally, smears scored as 4 showed abundant erythrocytes mostly in big clumps (>300 RBCs/40×).

The examination of the smear quality was also performed based on a rapid evaluation of the presence of endometrial cells, including those cells that exfoliate individually and those that were in clusters. These was generally categorized as the presence or absence of endometrial cells. The quantity of endometrial cells was evaluated in the cellularity category. Regarding endometrial cells, preservation was also graded by determining the presence of broken cells. A score of 0 or adequate was assigned when no broken cells were present, a score of 1 or good preservation was assigned when there were less than 25% broken cells, a score of 2 or fairly adequate was assigned when there were 25–50% broken cells, and a score of 4 or not useful for diagnosis was assigned when more than 50% of the endometrial cells were broken.

2.6.2. Cellularity and Cell Morphology

Epithelial endometrial cells were examined and scored from 0 to 4 (0 = no cells; 1 = very few cells; 2 = few cells; 3 = moderate number of cells; 4 = abundant cells), as previously described [28]. Cell morphology was determined by the observation of different features, including the degree of preservation (presence of pyknotic cells), disposition (individual, clusters, or acini formation), morphology (round, low-columnar, columnar), presence of cytoplasmic vacuoles or cilia, presence of a prominent nucleolus, and number of mitoses similar to that previously described in the bitch specimen [29].

2.6.3. Cell Ratios

Cell scores were used for the evaluation of the erythrocyte and leukocyte contents of the samples.

The presence of erythrocytes (RBC) was reported and ranged as described above in terms of the smear quality. Furthermore, an erythrocyte/endometrial cell ratio was calculated according to previously reported data [28] with slight modifications. Briefly, the ratio was assessed by determining the mean number of epithelial cells obtained by counting the number of epithelial cell clumps in 10 power fields at 400× magnification. Then, the mean was divided to obtain the erythrocyte score.

The presence of leukocytes was also evaluated and ranged from 0 to 4 (0 = no leukocytes; 1 = very few leukocytes; 2 = few leukocytes; 3 moderate number of leukocytes; 4 = abundant leukocytes) as previously described [28] with some modifications. A differential white blood cell count was also made from at least 100 leukocytes when possible.

2.6.4. Inflammation

The presence or absence of inflammation was made on basis of the PMN ratio according to previously defined criteria [30]. Briefly, 10 fields at 40× magnification were visualized and samples were scored in 4 categories: category 0 (no PMN); category +1 (1 to 10 PMNs individually distributed); category 2+ (more than 10 PMNs individually distributed); category 3+ (large clumps of PMNs). Preparations in category 0 were considered as not having inflammation, those in category 1 were considered to have mild inflammation, those in category 2 were considered to have moderate inflammation, and finally those in category 3 were considered to have severe inflammation.

2.7. Statistical Analysis

Variables were evaluated using the statistical package SPSS (version 22, IBM Corporation, Armonk, NY, USA). A Shaphiro–Wilk test was used to study the normal distribution of the variables. For those parameters with a non-parametric distribution, a Wilcoxson signed-rank test was applied to compare two related samples. Independent measurements were compared using the Mann–Whitney U test. For those parameters with a normal distribution, a two-way ANOVA test was used.

3. Results

3.1. Progesterone

Serum levels of progesterone ranged from 0.3 to 19.7 ng/mL. In UL queens the values ranged from 0.3 to 19.7 ng/mL, while in US queens they ranged from 0.8 to 3.0 ng/mL. Non-ovulated queens showed a mean value of 0.9 ± 0.1 ng/mL (ranging from 0.3 to 1.2 ng/mL) in the UL group and of 1.0 ± 0.1 ng/mL (ranging from 0.8 to 1.3 ng/mL) in the US group. Regarding the ovulated queens, mean serum progesterone values of 10.6 ± 1.3 ng/mL (ranging from 1.9 to 19.7 ng/mL) and 2.3 ± 0.5 ng/mL (ranging from 1.5 to 3.0 ng/mL) were obtained for the UL and US groups, respectively (Table 3).

Table 3. Serum levels of progesterone in the different evaluated groups. Values are expressed as means ± SEM (range).

Serum Progesterone (ng/mL)	Uterine Lavage	Uterine Swabbing
Ovulated	10.6 ± 1.3 (1.9–20.0) (n = 15)	2.3 ± 0.5 (1.5–3.0) (n = 6)
Non-ovulated	0.9 ± 0.1 (0.3–1.2) (n = 13)	1.0 ± 0.1 (0.8–1.3) (n = 16)

3.2. Microbiology

All samples yielded negative growth.

3.3. Histology

Histopathologic study of all samples did not identify any queens with signs of endometritis. Regarding the histological parameters studied, the results showed that the epithelial height in animals with high levels of progesterone (p_4 > 1.5 ng/mL) was significantly (p < 0.05) higher than those of animals with low levels of serum progesterone (p_4 < 1.5 ng/mL) (Table 4 and Figure 1).

Table 4. Results for histological examination. Values are expressed as the median plus the minimum and maximum.

	Low Progesterone			High Progesterone			
	Median	**Minimum**	**Maximum**	**Median**	**Minimum**	**Maximum**	***p***
Epithelial Height (cm)	7.95	5.22	11.40	15.23	6.36	31.22	0.000
Diameter 1 (cm)	53.99	28.03	89.47	86.97	40.00	158.33	0.039
Diameter 2 (cm)	37.67	22.33	54.57	53.04	23.39	90.36	0.039
Mean Diameter (cm)	45.82	25.18	68.74	70.00	36.10	124.35	0.012
Glandular Density (glands/400×)	6.12	0.95	11.40	7.49	0.50	18.20	0.278
Neutrophil (in 10 fields 400×)	0.49	0.00	4.60	0.16	0.00	0.60	0.053

There was no significant difference in the glandular density (p = 0.278) between groups (Table 4 and Figure 2). However, the glandular diameter was significantly (p = 0.012) increased in the high progesterone group compared with the low progesterone group (Table 4 and Figure 3).

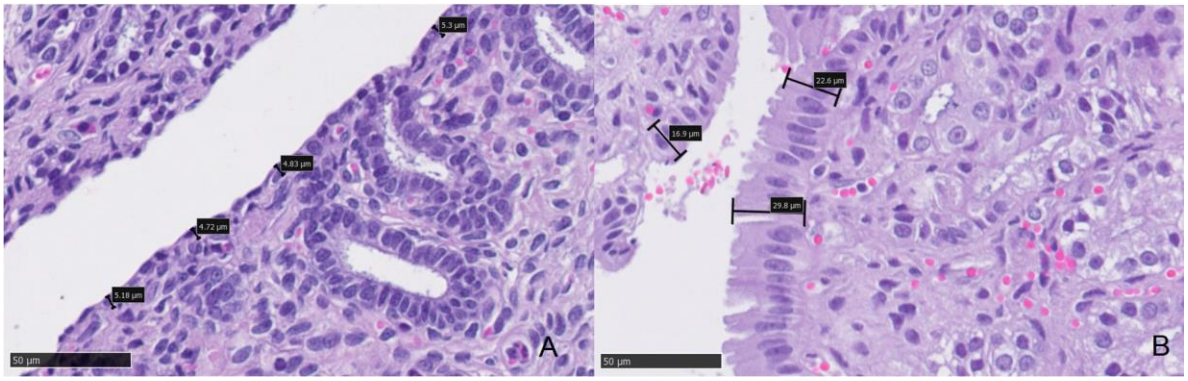

Figure 1. Epithelial height of a queen's endometrium. (**A**) Endometrial biopsy from a queen with low levels of serum progesterone (1.4 ng/mL). Measurements obtained in the luminal epithelium of the endometrium ranged from 4.72 to 5.3 mm (black bars and boxes). (**B**) Endometrial biopsy from a queen with high levels of serum progesterone (7.7 ng/mL). Measurements obtained in the luminal epithelium of the endometrium ranged from 16.9 to 29.8 mm (black bars and boxes). Endometrial epithelial height was significantly higher in queens with high levels of serum progesterone.

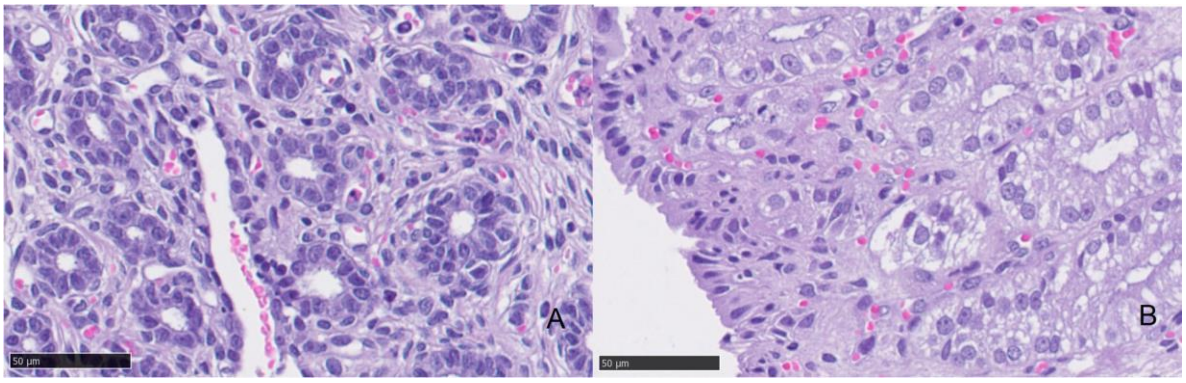

Figure 2. Glandular density in a queen's endometrium. (**A**) Endometrial biopsy from a queen with low levels of serum progesterone (0.8 ng/mL). (**B**) Endometrial biopsy from a queen with high levels of serum progesterone (7.7 ng/mL). Glandular density scores showed no statistical differences between low and high progesterone groups.

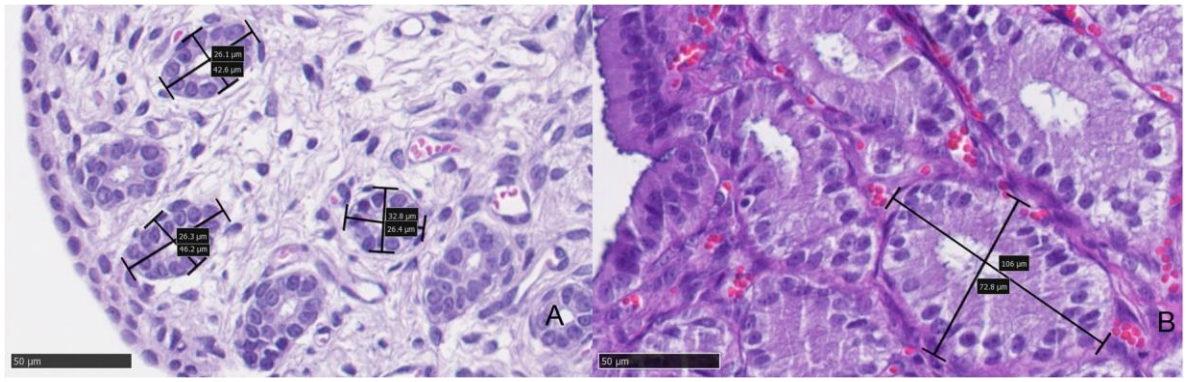

Figure 3. Glandular diameter in a queen's endometrium. (**A**) Endometrial biopsy from a queen with low levels of serum progesterone (0.8 ng/mL). Black bars and boxes show the measures taken from the two perpendicular diameters of the gland (from the basal lamina to the opposite one), with a total mean of 33.5 mm in that queen. (**B**) Endometrial biopsy from a queen with high levels of serum progesterone (6.5 ng/mL). Black bars and boxes show the measures taken from the two perpendicular diameters of the gland (from the basal lamina to the opposite one), with a total mean of 98.99 mm in that animal. The glandular diameter values were significantly increased in animals with high levels of serum progesterone compared with the low progesterone group.

Finally, the neutrophil counts were low in both groups and there were no statistically significant differences related to progesterone levels, although queens with high levels of progesterone showed a trend for lower neutrophil counts (Table 4 and Figure 4).

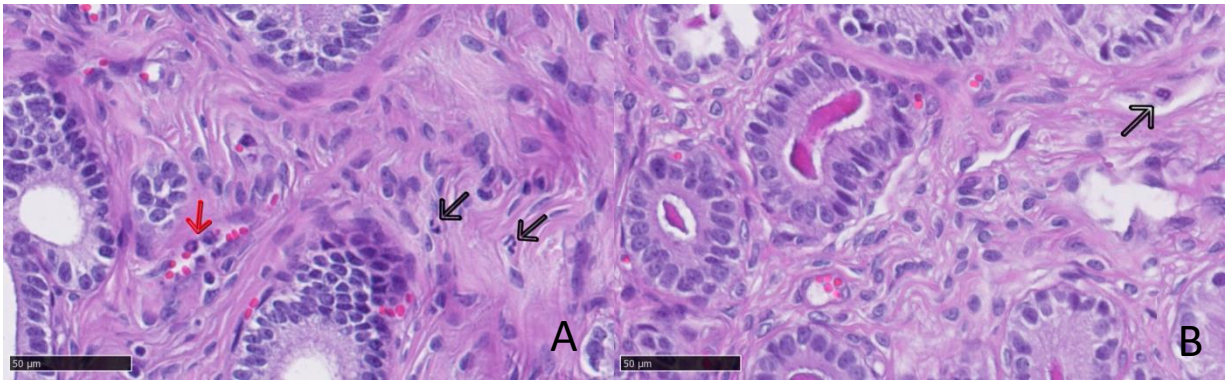

Figure 4. Presence of neutrophils in a queen's endometrium. (**A**) Endometrial biopsy from a queen with low levels of serum progesterone (0.25 ng/mL). Note: Two neutrophils admixed within the endometrium (black arrow); erythrocytes and neutrophils within a blood vessel (red arrow). (**B**) Endometrial biopsy from a queen with high levels of serum progesterone (19.7 ng/mL). One neutrophil admixed within the endometrium can be observed (black arrow).

3.4. Cytology

When evaluating the smear quality, cellularity, and morphology, although no statistically significant differences were observed between sampling methods, the preservation and cell morphology was detected to be moderately better in the samples obtained with the lavage method (Tables 5 and 6).

Table 5. Grading and distribution of the smear quality parameters according to the uterine sampling method. No statistically significant differences were observed for any of the evaluated parameters.

Smear Quality	Technique	Score 0	Score 1	Score 2	Score 3	Score 4	Comments
Mucus or Proteinaceous debris	Lavage	28 (100%)	0 (0%)	0 (0%)	0 (0%)	-	
	Swabbing	4 (18.18%)	18 (81.81%)	0 (0%)	0 (0%)	-	
Blood in Background (RBC)	Lavage	9 (32.14%)	8 (28.57%)	4 (14.29%)	4 (14.29%)	3 (10.71%)	
	Swabbing	0 (0%)	6 (27.27%)	6 (27.27%)	10 (45.45%)	0 (0%)	
Cellularity	Lavage	6 (21.42%)	9 (32.14%)	8 (28.57%)	5 (17.85%)	-	
	Swabbing	0 (0%)	10 (45.45%)	12 (54.54%)	0 (0%)	-	
Cell Preservation	Lavage	8 (34.78%)	6 (26.08%)	4 (17.39%)	5 (21.74%)	-	5 No cells
	Swabbing	0 (0%)	4 (18.18%)	12 (54.54%)	6 (27.27%)	-	
Epithelial Cells	Lavage	6 (21.42%)	10 (35.71%)	4 (14.29%)	4 (14.29%)	4 (14.29%)	
	Swabbing	0 (0%)	12 (54.54%)	6 (27.27%)	4 (18.18%)	0 (0%)	

Table 5. *Cont.*

Smear Quality	Technique	Score 0	Score 1	Score 2	Score 3	Score 4	Comments
Individual Epithelial Cells	Lavage	11 (39.29%)	8 (28.57%)	1 (3.57%)	5 (17.86%)	3 (10.71%)	
	Swabbing	0 (0%)	10 (45.45%)	10 (45.45%)	2 (9.09%)	0 (0%)	
Clusters Epithelial Cells	Lavage	7 (25%)	6 (21.43%)	7 (25%)	2 (7.14%)	6 (21.42%)	
	Swabbing	0 (0%)	2 (9.09%)	8 (36.36%)	10 (45.45%)	2 (9.09%)	

Table 6. Grading and distribution of the cytological characteristics of the cells in samples according to uterine swabbing or the uterine lavage method. No statistically significant differences were observed for any of these variables.

Cell Characteristics	Technique	Score 0	Score 1	Score 2	Score 3	Score 4	Comments
Acinar Arrangement	Lavage	18 (81.81%)	1 (4.55%)	3 (13.64%)	0 (0%)	-	5 No cells/1 not evaluable
	Swabbing	16 (72.72%)	4 (18.18%)	2 (9.09%)	0 (0%)	-	
Shape	Lavage	15 (68.18%)	3 (13.64%)	4 (18.18%)	-	-	5 No cells/1 not evaluable
	Swabbing	22 (100%)	0 (0%)	0 (0%)	-	-	
Pyknotic	Lavage	13 (59.09%)	9 (40.90%)	0 (0%)	0 (0%)	0 (0%)	5 No cells/1 not evaluable
	Swabbing	18 (81.81%)	4 (18.18%)	0 (0%)	0 (0%)	0 (0%)	
Cilia	Lavage	21 (95.45%)	1 (4.54%)	0 (0%)	0 (0%)	-	5 No cells/1 not evaluable
	Swabbing	22 (100%)	0 (0%)	0 (0%)	0 (0%)	-	
Vacuoles	Lavage	16 (72.72%)	3 (13.64%)	3 (13.64%)	0 (0%)	-	5 No cells/1 not evaluable
	Swabbing	22 (100%)	0 (0%)	0 (0%)	0 (0%)	-	
Nucleoli	Lavage	17 (77.27%)	2 (9.09%)	3 (13.64%)	0 (0%)	-	5 No cells/1 not evaluable
	Swabbing	20 (90.90%)	1 (9.09%)	1 (9.09%)	0 (0%)	-	
Mitosis	Lavage	21 (95.45%)	1 (4.54%)	0 (0%)	0 (0%)	-	5 No cells/1 not evaluable
	Swabbing	22 (100%)	0 (0%)	0 (0%)	0 (0%)	-	

Regarding cell scores, only the RBC/endometrial cell ratio and white blood cells (WBC) count were significantly different between methods (Figures 5–7), being higher in samples obtained by uterine swabbing than those obtained by uterine lavage.

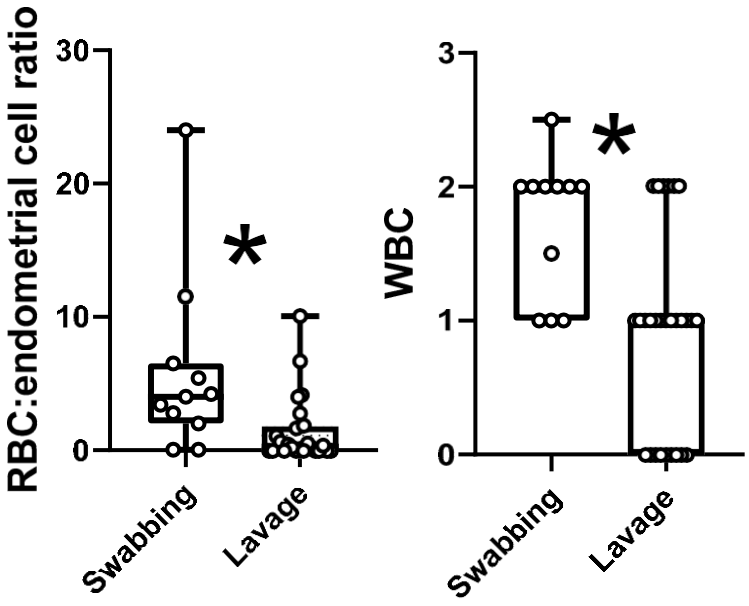

Figure 5. Red blood cells (RBCs): Endometrial cell ratio and white blood cell (WBC) numbers. * Samples obtained by uterine swabbing showed statistically significant ($p < 0.05$) higher values for these two analyzed parameters in comparison with samples obtained by uterine lavage.

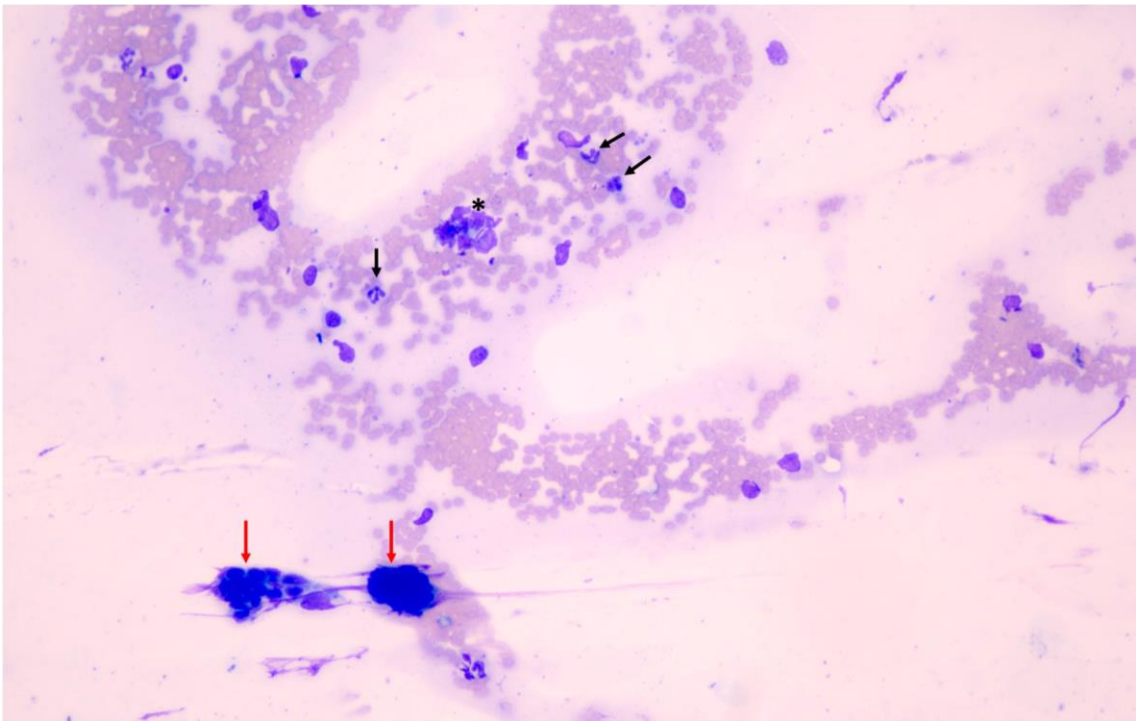

Figure 6. Endometrial cytology results obtained using the swabbing technique (200× magnification). Note the presence of abundant red blood cells (RBCs), which increases the RBC/endometrial cell ratio and the white blood cells content of the smear. Black arrows indicate the presence of neutrophils. Red arrows indicate clusters of endometrial cells. The asterisk indicates a broken cell.

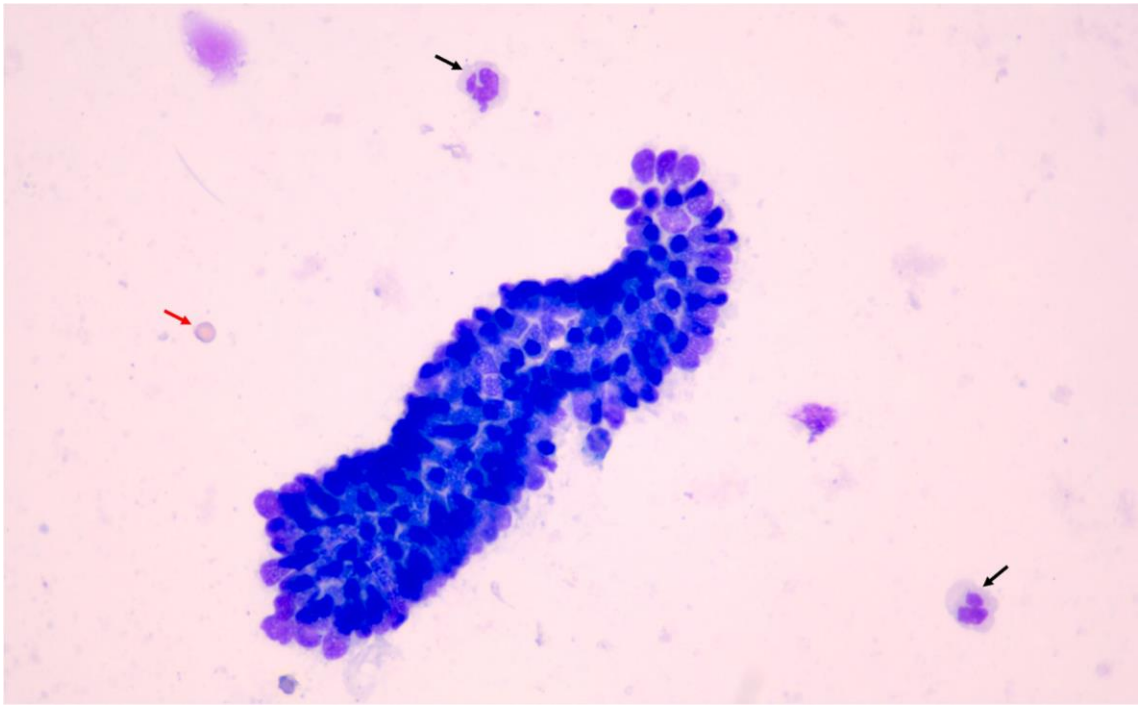

Figure 7. Endometrial cytology results obtained using the lavage technique (400× magnification). A big cluster of endometrial cells was detected and cell morphology was well preserved. Note the low numbers of red blood cells (red arrow) and leukocytes (black arrows).

Furthermore, the numbers of PMN cells were not statistically significant between the evaluated methods (Table 7), although increased percentages of swabbing samples were classified as category 2+. In swabbing samples, 9% of the samples were classified as category 0, 36% as category 1, 50% as category 2, and 5% as category 3. In lavage samples, 32% were classified as category 0, 39% as category 1, 29% as category 2, and no samples were classified as category 3 (Table 7).

Table 7. Total numbers of cases and percentages per category for polymorphonuclear neutrophils (PMN) using each method. Category 0 corresponds to the absence of polymorphonuclear neu- trophils (PMN) in 10 fields at 40× magnification. Category 1+ corresponds to a total count of 1 to 10 PMNs. Category 2 corresponds to a PMN count >10, which are individually dispersed. Category 3+ corresponds to a PMN count >10, which are distributed in clumps.

	Category 0	Category 1+	Category 2+	Category 3+
Swabbing	2 (9%)	8 (36%)	11 (50%)	1 (5%)
Lavage	9 (32%)	11 (39%)	8 (29%)	0 (0%)

Finally, regarding inflammation, none of the samples showed increased numbers of neutrophils so as to suggest endometritis, regardless of the sampling group.

When different levels of progesterone were considered (i.e., ovulated versus non-ovulated queens), no significant differences in the cytological analysis were observed between sampling groups.

4. Discussion

Endometritis diagnosis is performed by combining different diagnostic tools such as biopsy, cytology, and culture, among others, with histology classically being used as a fundamental tool for several mammalian species [18–21]. Histopathological findings have been used to categorize the severity of endometritis in bitches [20]. Animals with neutrophilic or eosinophilic infiltration are classified as having acute endometritis, those

with mixed infiltration are classified as having subacute endometritis, while animals that have lymphocytic or lymphoplasmocytic infiltration are classified as having chronic endometritis. However, literature related to the study of endometrium and endometritis in queens is scarce.

Knowledge of normal uterine characteristics in a healthy subjects is mandatory to detect alterations that can contribute to fertility problems. In the present study, microbi- ological cultures and biopsies were used as controls to establish the actual status of the endometrium. All microbiological cultures yielded negative results and PMN counts were low in all of the samples, which allowed all of the analyzed endometria to be categorized as normal.

In addition, the endometrial structure was evaluated in the present study and modifications related to serum levels of progesterone were described. The epithelium and stroma undergo cyclic morphological and biochemical changes during the reproductive cycle, which are necessary to create the optimal environment for the survival of the fetus [31–38]. These changes are thought to be secondary to the actions and fluctuations in serum steroid hormones levels. In queens, three different histological patterns of the uterus (inactive phase, follicular phase, and luteal phase) according to the stage of the queen's reproductive cycle have been previously described by Chatdarong et al. [39]. In that study, epithelial luminal height was significantly higher during the luteal phase (high progesterone and low estradiol serum levels) than during the inactive phase (low progesterone and estradiol serum levels), but not during the follicular phase (low progesterone and high estradiol serum levels). These results are in agreement with the present study, in which we observed increases of the epithelial height and the glandular diameter under the influence of high levels of serum progesterone. In our study, queens were classified according to serum progesterone levels, while estradiol levels were not considered, which could be a limitation, as we did not identify animals in the follicular phase or confirm than the epithelium heights did not differ from animals with high progesterone levels. Additionally, punctual determination of serum estradiol levels would not be representative of the reproductive status of a queen. Serialized blood sampling for hormone determination would be a more accurate way to determine the role of steroid hormones in the endometrium of a queen throughout the sexual cycle. Since the queens included in the study belonged to a neutering program, serialized blood sampling was beyond the authorized permission.

To the authors' knowledge, this is the first study wherein histology includes the evaluation of the glandular density and glandular diameter in order to further characterize the histologic appearance of queens in different phases of the reproductive cycle. Our results agree with those previously observed in mares on day 7 of pregnancy [40]. Specifically, increased glandular diameter, height of the glandular epithelium, and glandular lumen were observed in 7-day-pregnant mares in comparison with cyclic mares. All of these changes have been observed to appear in concomitance with the entry of the embryo into the uterus. Focusing on the increased height of the luminal epithelium and glandular diameter, these changes have been suggested to be related with the production of glandular secretions, which are released into the uterine lumen during early pregnancy.

On the other hand, cytological examination of the endometrium is considered a useful technique and has gained importance as a part of the diagnosis of endometritis in different species, such as bitches, mares, and cows [1,10,41]. In the literature, different techniques have been described for the collection of endometrial and inflammatory cells. Uterine lavage and swabbing techniques are the most accepted cytological methods in cows, since they are less invasive [42]. Although histology is still considered the most reliable tool for diagnosing endometritis in many species such as mares [18,19], bitches [20] or cows [21], the reduced endometrium size of queens makes it difficult to obtain representative samples. Having a minimally invasive cytology technique that yields preserved endometrial and inflammatory cells of the endometrium surface would provide a more feasible tool for endometritis diagnosis.

In the present study, samples obtained with UL and US methods were evaluated to determine their applicability in order to obtain cellular samples from the feline endometrium. Furthermore, both techniques were compared to evaluate which one was more appropriate for establishing the actual status of the feline endometrium. Both sampling techniques collected enough endometrial cells to consider them as diagnostic. However, swabbing samples showed statistically significant higher RBC/endometrial cell ratios and higher WBC counts compared with lavage samples, with lavage samples closer to histological results than swabbing samples. These differences are probably due to the friction of the endometrial surface with the swab during the sampling, causing the rupture of superficial vessels and facilitating bleeding. The bleeding probably contributed to the increase of WBC percentages, as well as the higher RBC/endometrial cell ratios obtained. The present results demonstrate that endometrial smears obtained by uterine lavage are more reliable for establishing the status, at least when healthy, of the feline endometrium.

Although no significant statistical differences were observed, when evaluating the smear quality, cellularity, and morphology, cell preservation and morphology results were detected to be moderately better in the samples obtained with the lavage method. Furthermore, samples obtained with the US method showed higher scores in the PMN evaluation, although the differences were not sufficient to be statistically significant.

As previously stated, the aim of the present study was to determine which one of the sampling techniques (US vs. UL) was more accurate. It is also important to keep in mind the fact that while uterine lavage is a clinically applicable technique in queens [43], uterine swabbing is not.

In contrast with observations of histology, cytology results from the present study did not show any variation in the cell exfoliation degree or in the cell morphology according to serum progesterone levels.

The study has some limitations. First of all, queens included in this study were feral and information on their previous reproductive history, age, and reproductive status was not available. Another limitation was that none of the queens included in the study had endometritis; thus, further research including queens with endometritis is warranted to establish the real utility of endometrial cytology in pathological conditions.

5. Conclusions

In conclusion, although further studies including queens with endometritis are warranted, the combination of endometrial cytology and uterine culture could be used to evaluate the endometrial status of queens. The uterine lavage method is less affected by blood contamination, cells are better preserved, and the samples are more representative of reality when compared with US samples. Although serum progesterone did not affect the cytology results, histologically the present results confirm previous reported data on the increase of endometrial epithelium height. In addition, the glandular diameter was also increased in queens with high levels of serum progesterone. Endometrial remodeling is probably necessary for the proper implantation and development of pregnancy.

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Conflicts of Interest: The authors declare no conflict of interest.

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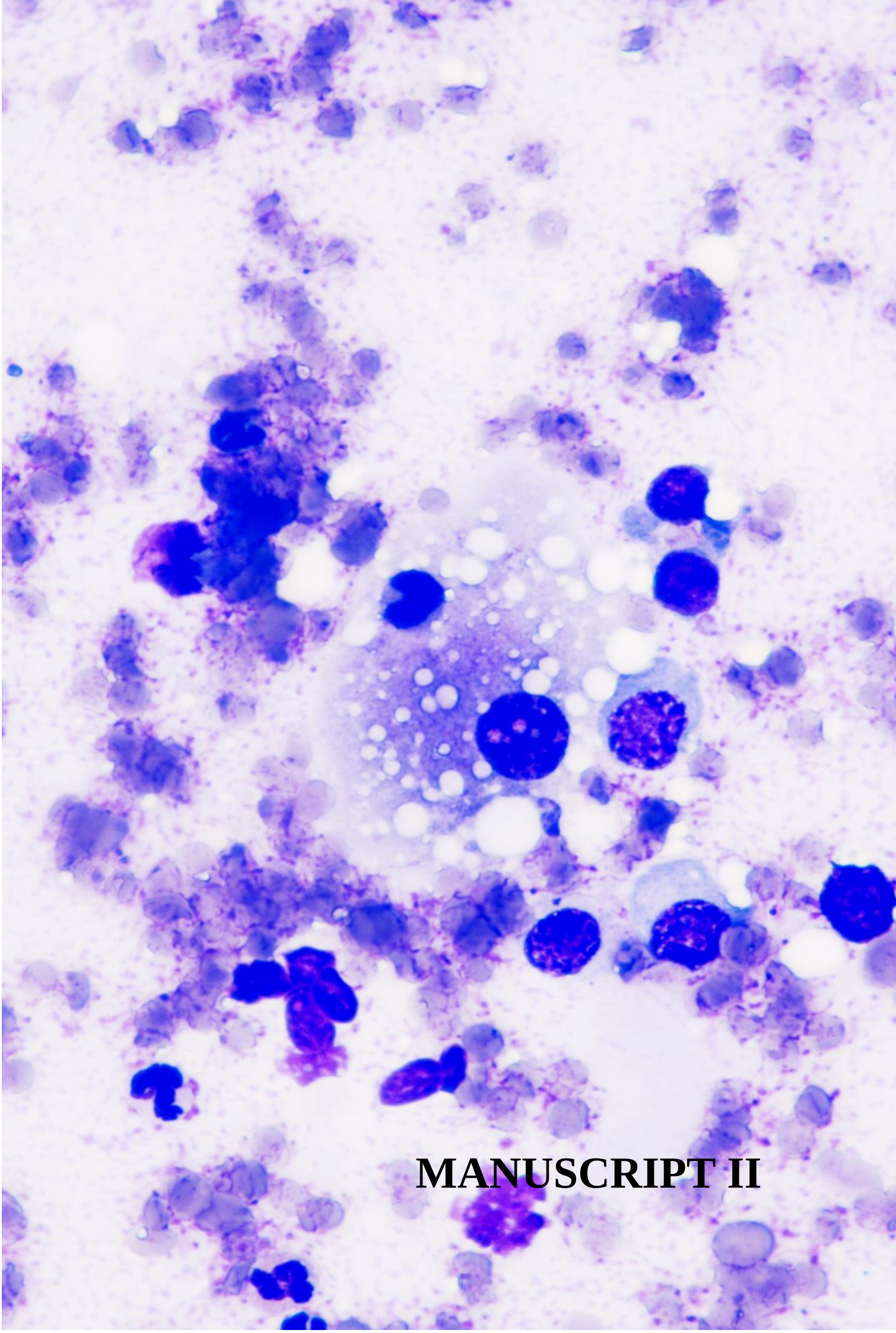

MANUSCRIPT II

VIII. MANUSCRIPT II

Cytological characteristics of the feline ovary at different sexual stages

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Abstract

Cytology is a valuable diagnostic tool in veterinary medicine, allowing rapid and minimally invasive diagnosis. However, its application in the reproductive tracts of small animals is limited, mainly assessing mammary masses and staging the oestrous cycle. This study evaluated the cytological characteristics of healthy queen ovaries throughout the oestrous cycle. Twenty-four domestic shorthair queens were included and submitted to a conventional ovariohysterectomy. A blood sample was obtained to assess the serum concentration of oestradiol and progesterone. One ovary was immersed in paraformaldehyde for histology purposes, whereas the other was used for cytology. Blood parameters and histology were used to classify the queens into 4 different groups: anestrous, follicular, corpus luteum (CL) and luteal-cell groups. When comparing the groups, statistically significant ($P<0.05$) differences were observed only in the percentage of luteal and mesenchymal cells and mitotic figures. Luteal cells were only present in those ovaries belonging to the CL group. Mesenchymal cells were present in the follicular and the CL groups. Mitotic figures were observed in the anestrus and follicular phases. It can be concluded that the ovarian cytology in the queen varies according to the reproductive cycle.

Keywords: ovary; corpus luteum; ovarian follicles; cytology; histology

1. Introduction

The ovary is an "always-changing" structure. Both anatomy and endocrine functions are modified under the influence of follicle-stimulating (FSH) and luteinising (LH) hormones. Ovaries carry two main tasks, the production, differentiation and release of female gametes, and the production of hormones involved in the reproductive functions of the female, such as sexual cyclicity, pregnancy and parturition (Senger, 1999).

Anatomically, the ovary is divided into two main structures, namely (1) the ovarian medulla, which houses the vasculature, the innervation and lymphatic vessels; and (2) the ovarian cortex, which houses the population of oocytes, corpora lutea (CL) and corpora albicantia (Senger, 1999). Oocytes are surrounded by follicular cells, thus composing the ovarian follicles. The size and shape of the ovarian follicles will vary depending on the follicular development and maturity stage. According to this, the types of follicles observed in the ovary are primordial, primary, secondary and tertiary or antral. Primordial follicles comprise the oocyte and a single layer of surrounding flattened cells. Primary follicles also present a single layer of cells surrounding the oocyte, but they are cuboidal cells. Secondary follicles present two or more layers but without an antrum. Finally, tertiary follicles present several layers of surrounding cells and a fluid-filled antrum (Senger, 1999).

Once ovulation occurs, the follicle collapses, and theca interna and granulosa cells undergo luteinisation (Havelock et al., 2004; Magoffin et al., 2005). The CL is composed of luteal cells, leukocytes, fibroblasts, endothelial cells and pericytes (Stocco et al., 2007). Its morphology has already been described in detail in several species such as Canadian porcupine, ovine, porcine, bovine and human (Mossman and Judas, 1949; Thwaites and Edey, 1970; Fitz et al., 1982; Hehnke et al., 1994; Fields and Fields, 1996; Duncan, 2000). The queen is not an exception, and ovarian morphology has been recently described and updated using imaging diagnosis techniques (Gatel et al., 2020) and histology (Jewgenow et al., 2012; Amelkina et al., 2015).

Cytology is a recognised technique in human medicine that is specially used to obtain preoperative cytodiagnosis of ovarian masses (Pal et al., 2015). In veterinary species, cytology is commonly used to evaluate various tissues. In large animals like mares and cows, the cytology of the endometrium is frequently used to assess the reproductive health of the females. However, in small animals like dogs and cats, the use of cytology in the reproductive tract is much more limited. In that species, the inaccessibility of the reproductive tract makes it challenging for the clinician to obtain reliable diagnostic

information. In dogs and cats, vaginal cytology is a highly recognised tool to stage the oestrous cycle. Likewise, uterine cytology and biopsy samples can be obtained, but these procedures are more invasive in small animals than in other species and require higher expertise (Wilborn and Maxwell, 2012). The ovaries are also very difficult to reach, and little is known about ovarian cytology. In dogs, some authors have described the specific cytologic features of the normal ovary (Piseddu et al., 2012), with cytology being considered a relevant tool for detecting ovarian neoplasia (Bertazzolo et al., 2004; Solano-Gallego, 2023). However, according to the authors' knowledge, cytological findings of the queen ovary obtained by fine needle aspiration remain unknown.

Thus, the present study aimed to provide a cytologic description of ovarian aspirates from queens at different sexual phases, and to compare the findings with those from histological examination of the ovaries and serum concentration of steroid sexual hormones.

2. Materials and Methods

2.1. Animals

The present study included twenty-four domestic short-hair queens from a "trap, neuter and return" program carried out by the Surgery Unit at the Veterinary Hospital of the Universitat Autònoma de Barcelona (UAB). All the included females were stray queens, thus impeding the obtaining of information about previous reproductive history, reproductive status and age. Samples were obtained from November to December and from February to March.

Queens were included in the study if (1) the physical examination was considered normal and (2) they tested negative against feline leukaemia virus and feline immunodeficiency virus (Snap FIV/FeLV; Idexx Laboratories, Westbrook, ME, USA).

Before the surgery, queens were sedated using an intramuscular combination of 5mg/kg Ketamine (Ketamidor®, Richter pharma, Austria) with 20μg/kg buprenorphine (Buprecare®, Divisa FarmaVic, S.A, Spain) and 0.2 mg/kg midazolam (Midazolam®, Normon S.A, Spain). After sedation, a blood sample from the jugular vein was collected and placed into a glass tube. The blood was allowed to clot and then centrifuged at 2200 x g for 10 minutes at room temperature. The serum was immediately frozen and kept at -20ºC until analyses were performed.

Queens were then induced with 2-4 mg/kg propofol (Propovet TM, Zoetis Ecuphar, Spain). The anaesthetic status was maintained using 1.5-2% isoflurane (IsoFlo, Zoetis

Ecuphar, Spain) in oxygen using a Mapleson F anaesthetic breathing circuit and a conventional ovariohysterectomy through ventral midline laparotomy was performed.
The study was approved by the UAB's Ethical Committee of Animal Care and Research (CEEAH, code 2939).

2.2. Blood samples

Blood samples were used to determine the serum concentration of oestradiol and progesterone. Oestradiol concentration was determined using the Estradiol sensitive ELISA commercial kit (Demeditec Diagnostics GmbH, Kiel, Germany) and according to the manufacturer's instructions. The detection limit ranged from 1.40 to 200 pg/mL. Intra-assay coefficients of variation were 7.87%, 5.68% and 5.52% for 8.21 pg/mL, 18.50 pg/mL and 27.62 pg/mL, respectively. Inter-assay coefficients of variations were 8.78%, 7.25% and 6.78% for 7.87 pg/mL, 17.87 pg/mL and 26.71 pg/mL of estradiol, respectively. Those queens showing estradiol concentration above 20 pg/mL were considered to be undergoing the follicular phase (Shille et al., 1979).
The serum concentration of progesterone was determined using the Immulite 1000 equipment (Immulite; Siemens Healthcare Diagnostics, Cornellà del Llobregat, Spain). The detection limit of the equipment ranged from 0.2 to 40 ng/mL. Intra and inter-assay coefficients of variation of the instrument were 5.5 and 6.5, respectively. Queens with serum concentrations of progesterone below 1.5 ng/mL were considered non-ovulated, whereas those showing serum concentrations above 1.5 ng/mL were considered ovulated (Paape et al., 1975; Verhage et al., 1976; Wildt et al., 1981; Schmidt et al., 1983).

2.3. Cytology

After the surgical removal of the reproductive tract, fine-needle aspiration (FNA) of one of the ovaries was performed using a 25-gauge needle and a 5-mL syringe. The needle was introduced inside the ovary, opposite to the ovarian hilum, and was randomly redirected multiple times to obtain ovary specimens. After the cytology sampling, the material obtained was squashed on a glass slide and allowed to air dry, thus obtaining two smears from each queen (n = 48 smears). Since the FNA technique may modify the physiological architecture of the ovaries, the histological assessment was performed with the contralateral ovary.
Dried samples were stained with a modified Giemsa stain (Auto-Hemacolor®, Merck KGaA, Darmstadt, Germany) using an automatic dye (Hematek®, Siemens Healthcare

Diagnostics Inc., Tarrytown, NY, USA). Smears were examined under a light microscope at 100x to 1000x magnification (Nikon Eclipse Ci, Japan) by a board-certified pathologist and a resident of clinical pathology separately. Both pathologists performed a blind assessment of the samples. A consensual result was obtained after the potential discrepancies were revised. Smears were evaluated at low magnification (low power field (LPF)=100x) to assess general features related to the quality of the smear, such as the background, the cellularity and the cell preservation, the type of cells encountered, and the blood contamination. After that, cytologic specimens were evaluated at higher magnification (high power field (HPF)=600x) to determine the cell arrangement, the characteristics of the specific cell types and the presence of mitotic figures. A higher magnification (1000x) was applied when a more defined detail of the nuclear and/or cytoplasmic characteristics was necessary. Finally, the number and distribution of leukocytes were also evaluated at high magnification (400x) (adapted from Piseddu et al., 2012). The main evaluated parameters are summarised in Table 1.

2.3.1 Background

The background evaluation included debris presence, fat-droplet content, extracellular matrix or pink proteic material and artefacts presence (Table 1). The presence of debris was graded from 0 to 3, being graded as 0 for those smears with a clear background without debris, 1 for those preparations with some debris present, 2 for preparations with abundant debris but still adequate for diagnosis, and 3 for those with abundant debris and not adequate for diagnosis.

Fat droplets were graded from 0 to 4. A 0-score was given if no fat droplets were present, a 1-score was for the preparations with scattered fat droplets, 2 for those with few droplets, 3 if a moderate number of fat droplets were present and 4 if they were abundant.

The presence of extracellular matrix was also evaluated and graded from 0 to 3. A score of 0 was given when no material was found, 1 was given to preparations with scattered matrix, 2 to those with a moderate quantity of matrix, and 3 to those preparations showing abundant matrix.

Finally, the presence of artefacts, such as stain material, was evaluated using a score from 0 to 2. Smears graded with a 0 had no artefact present. Smears graded with 1 had some artefact but were still acceptable for diagnosis. Finally, smears with a grade of 2 had excessive artefacts and were considered unsuitable for diagnosis.

Table 1. Cytologic features evaluated in the ovary smears. AD: Adequate for diagnosis; NAD: Not adequate for diagnosis; LPF: low power field, 400x magnification; HPF: high power field, 600x magnification.

Cytological Feature	**Score**				
	0	**1**	**2**	**3**	**4**
Background					
Debris in the background	Clear background	Debris present (AD)	Abundant debris	NAD	-
Fat droplets	Absent	Scattered	Few	Moderate	Abundant
Extracellular matrix	Absent	Scattered	Moderate	Abundant	-
Artifacts	Absent	Scattered (AD)	Excessive (NAD)	-	-
Cellularity/cell preservation					
Cellularity (cells/LPF)	Absent (0)	Scattered (1-50)	Moderate (51-100)	Abundant (>100)	-
Cell distribution/overlapping	Uniform Minimal/no overlapping	Restricted to one area Minimal/moderate overlapping	Clumps through a slide Moderate/abundant overlapping	Clumps, very overlapped or in only a few areas of cells	-
Cell preservation (% of broken cells)	Good (0%)	Adequate (<25%)	Moderately adequate (25-50%)	Poor (>50%)	-
Free nuclei (% of cells)	None	Scant (1-40%)	Moderate (40-80%)	Abundant (>80%)	
Cell arrangement (number/LPF)					
Individual Epithelial Cells	Absent (0)	Scant (<10)	Few (10-40)	Moderate (41-80)	Abundant (>80)
Clusters Epithelial Cells	Absent (0)	Scant (<10)	Few (10-40)	Moderate (41-80)	Abundant (>80)
Acinar arrangement	Absent (0)	Scattered (1)	Few (2-3)	Moderate (>3)	-
Palisade arrangement	Absent (0)	Scattered (1)	Few (2-3)	Moderate (>3)	-
Papillary arrangement	Absent (0)	Scattered (1)	Few (2-3)	Moderate (>3)	-
Specific cell type					
Luteal cells (cells/LPF)	Absent (0)	Scattered (<10)	Few (10-20)	Moderate (21-50)	Abundant (>50)
Granulosa cells (cells/LPF)	Absent (0)	Scattered (<10)	Few (10-20)	Moderate (21-50)	Abundant (>50)
Multinucleated (cells/LPF)	Absent (0)	Scattered (<10)	Few (10-20)	Moderate (21-50)	Abundant (>50)
Mesenchymal cells (cells/LPF)	Absent (0)	Scattered (<10)	Few (10-20)	Moderate (21-50)	Abundant (>50)
Mitosis (number of mitosis/HPF)	Absent (0)	Scattered (1)	Moderate (2-3)	Abundant (>3)	-

2.3.2 Cellularity and cell preservation

Cellularity and cell preservation were evaluated according to the number of cells, the cell distribution and overlapping, the preservation of the cells and the presence of free nuclei (Table 1). Cellularity was scored from 0 to 3. A score of 0 was given when no cells were found. A score of 1 if scattered cells were present (1-50 cells/ LPF). A score of 2 if a moderate number of cells were found (50-100 cells/LPF) and finally 3 if the preparation had abundant cellularity (>100 cells/LPF).

Cell distribution and overlapping were also evaluated and graded from 0 to 3. A score of 0 was given to the preparations that were uniformly distributed with none or only minimal overlapping. A score of 1 was given to those samples where cells had minimal to moderate overlapping or if they were found in restricted areas. A score of 2 was given to samples showing clumped areas and moderate to abundant cell overlapping. Finally, a 3 score was assigned to those preparations with highly overlapped cells or cells distributed only in clumps or for preparations with few areas of cells, making them unsuitable for diagnosis.

Cell preservation was also graded from 0 to 3. A score of 0 was given to preparations with perfect cell preservation, thus containing 0% of broken cells. Score 1 was considered good preservation and was given to those preparations presenting less than 25% of broken cells. Score 2 was given to those smears that showed moderately adequate preservation and contained 25 to 50% of broken cells. Finally, a 3 score was given to those preparations with more than 50% of the cells broken and useless for diagnosis.

Finally, the presence of free nuclei was also evaluated and graded from 0 to 3. A score of 0 was given to the preparations with an absent free nucleus. A score of 1 was given when there were scant free nuclei (1-40% of cells), a score of 2 when there were moderate numbers of free nuclei (40-80% of the cells) and a score of 3 when there were abundant numbers of free nuclei (>80% of the cells).

2.3.3 Cell arrangement

Cell arrangement was evaluated according to the number of individual cells and clusters present and the specific type of arrangement (acinar, palisade and papillar) (Table 1). Individual cells and clusters were graded from 0 to 4. A score of 0 was given when no individual cells nor clumps were found, 1 for preparations with <10 individual or cluster cells/LPF, 2 for preparations with 10-40 individual or cluster cells/LPF, 3 for preparations with 40-80 individual or cluster cells/LPF and 4 for those with >80 cells/LPF.

The specific type of arrangement was evaluated and graded separately from 0 to 3 for each type. A score of 0 was determined when the particular type of cell arrangement was absent, 1 when there were scattered cells of the specific type (<1/600x), 2 when few cells of the particular type of arrangement were present (1-3/600x) and 3 for the moderate presence of the specific type of arrangement (>3/600x).

2.3.4 Specific cell type

Cell type assessed the presence of recognisable specific cells such as luteal, granulosa, and mesenchymal cells. The presence of mitotic figures was also evaluated. Furthermore, large multinucleated structures were occasionally present in some of the samples, and their presence was also graded (Table 1).

The presence of the different specific cell types was evaluated separately and graded from 0 to 4. A score of 0 was given when the smear did not contain any specific type of cell. A score of 1 was given when <10 cells/LPF were encountered, a score of 2 was given when 10-20 cells/LPF were found, and a score of 3 was given when 20-50 cells/LPF were found. Finally, a score of 4 was given when >50 cells/LPF were encountered.

Finally, the presence of mitotic figures was graded from 0 to 3. A 0 score was given when no mitotic figures were found, a 1 score was for the preparations that contained 1-3 mitotic figures/600x and a score of 3 was given when >3 mitotic figures/600x were found.

2.3.5 Presence of erythrocytes and leukocytes

The presence of erythrocytes and leukocytes in the background was evaluated and graded following a similar approach as previously described by Piseddu et al. (2012). The scores are summarised in Table 2.

A numerical score from 0 to 3 was used to classify smears according to the degree of blood contamination. Smears with a score of 0 showed an absence of blood contamination. Smears scored with 1 contained mild blood contamination. Smears with a 2-score showed moderate blood contamination. Finally, the smears with a score of 3 showed marked blood contamination.

The number of leukocytes was determined by evaluating 10 fields at high magnification (400x) and was scored from 0 to 3. A score of 0 was given to the smears that had no leukocytes. Smears scored with 1 showed sporadic leukocytes (1-3/400x). A score of 2 was given to smears that presented moderate numbers of leukocytes (4-6/400x). Finally, the smears with a score of 3 had a high number of leukocytes (7-10/400x). The leukocyte score was then compared with the erythrocyte score to determine the impact of blood contamination. If the leukocyte score was the same or lower than the erythrocyte score, the leukocyte presence was attributed to blood contamination. If the leukocytes were admixed with the ovarian cells, their presence was considered relevant in the tissue and not attributed to the blood contamination.

Table 2. Presence of erythrocytes and leukocytes.

Cytological Feature	**Score**			
	0	**1**	**2**	**3**
Erythrocyte content (cells/HPF)	Absent	Mild blood content	Moderate blood content	Marked blood content
Leukocyte content (cells/HPF)	Absent	Sporadic (1-3)	Occasional (4-6)	Moderate numbers (7-10)

HPF: high power field (400x)

2.4. Histology

A histological assessment was performed to corroborate the cytological findings. For that purpose, the ovary not used for cytology sampling was placed in 10% paraformaldehyde immediately after surgery to avoid the possible alterations in the ovarian architecture caused by cytology sampling. Samples were embedded in paraffin blocks, and 4 μm-thick sections were obtained and stained with hematoxylin-eosin. Stained sections were scanned and viewed using the NDP View 2 software (Hamamatsu Photonics, Korea). The biopsy examination consisted of the preliminary evaluation of the presence or absence of ovarian follicles and luteal tissue. Luteal tissue was classified into two different categories, namely CL and luteal-cell phases. The CL phase comprised those ovaries containing structurally defined CL, whereas the luteal-cell phase comprised those ovaries which did not contain structurally defined CL, but luteal cells were present.

2.5. Queens classification

Queens were divided into four groups, namely the anestrous, the follicular, the CL (either pregnant or pseudopregnant) and the luteal-cell phases, according to the hormonal concentration and the ovarian histological morphology. The anoestrus group included those queens presenting low serum progesterone and estradiol concentration, with no apparent CL or few amounts of luteal cells. The follicular phase group included queens presenting serum estradiol concentration above 20 pg/mL, serum concentration of progesterone below 1.5 ng/mL and no apparent CL or few amounts of luteal cells. CL group included queens presenting serum concentration of progesterone above 1.5 ng/mL, regardless of the presence of an evident pregnancy (pregnant queens) or not (pseudopregnant queens), and evident CL. Finally, the luteal-cell phase included those

queens presenting high serum concentrations of progesterone with no apparent CL or few amounts of luteal cells [19-23].

2.6. Statistical analysis

Queens' CL life span was histologically divided into four groups according to histological characteristics and hormonal concentration. The four groups were anoestrus, follicular, CL, and luteal-cell phases. The chi-square test was used with Fisher's multiple comparison post hoc to compare the different groups. The statistically significant difference was set at $p<0.05$. Statistical package SPSS (version 22, IBM Corporation, United States) was used for the analysis.

3. Results

3.1 Queens classification

Fifteen out of the 28 queens were sampled between November and December. The other 9 females were sampled between February and March (Table 3). According to hormonal concentration and histological appearance of the ovaries, 5 animals were classified in the anoestrus group, 7 queens were included in the follicular group, 8 queens were classified in the CL phase, and 4 were classified as the luteal-cell phase. Six out of the seven queens included in the luteal phase group were found to be pregnant.

3.2 Blood samples

The serum concentrations of progesterone and oestradiol are shown in Figures 1A and B. Blood samples from three pregnant queens in the luteal phase group were missing. For statistical purposes, progesterone values below the detection limit were considered 0.20 ng/mL (n=5), values above the detection limit were considered 40 ng/mL (n=2), and oestradiol values below the detection limit were considered 1.4 pg/mL (n=6) (Table 3).

Progesterone concentration was significantly higher in the luteal phase group (8.79±2.27 ng/mL). Samples from follicular and anoestrus phases showed serum concentrations of progesterone below 1 ng/mL. Finally, progesterone values in queens from the luteal-cell phase ranged between 1.7 and 3.49 ng/mL, with a mean value of 2.46±0.99 ng/mL.

Regarding oestradiol, only queens from the anoestrus group showed negligible values of this hormone. Follicular, luteal and luteal-cell phases all showed high mean values of oestradiol.

Table 3. Queens classification according to histology and hormonal concentrations.

Animal	Sampling period	Group	Histology F/CL	Oestrogens (pg/mL)	Progesterone (ng/mL)
1	November	Follicular	+/-	70,568	0,2
2	November	Follicular	+/-	24,564	0,2
3	November	Follicular	+/-	138,393	0,708
4	November	Follicular	+/-	26,244	0,737
5	November	Follicular	+/-	13,646	0,71
6	November	Follicular	+/-	145,393	0,726
7	December	Follicular	+/-	63,832	0,511
8	March	Luteal	+/+	0,1	11
9	March	Luteal	+/+	NA	NA
10	March	Luteal	+/+	11,176	11,4
11	February	Luteal	+/+	56,008	6,52
12	February	Luteal	+/+	NA	NA
13	February	Luteal	+/+	1,4	8,02
14	March	Luteal	+/+	NA	NA
15	November	Luteal	+/+	56,18	6,99
16	December	Luteal-cell	+/±	10,256	1,7
17	February	Luteal-cell	+/±	116,126	3,12
18	November	Luteal-cell	+/±	1,4	1,52
19	November	Luteal-cell	+/±	38,149	3,49
20	March	Anoestrus	+/-	0,492	0,203
21	November	Anoestrus	+/-	1,4	0,2
22	November	Anoestrus	+/-	1,4	0,2
23	November	Anoestrus	+/-	1,4	0,2
24	November	Anoestrus	+/-	1,4	0,666

NA: Samples not available; F: follicles; CL: corpus luteum; +: presence; -: absence; ±: luteal cells with no CL structure.

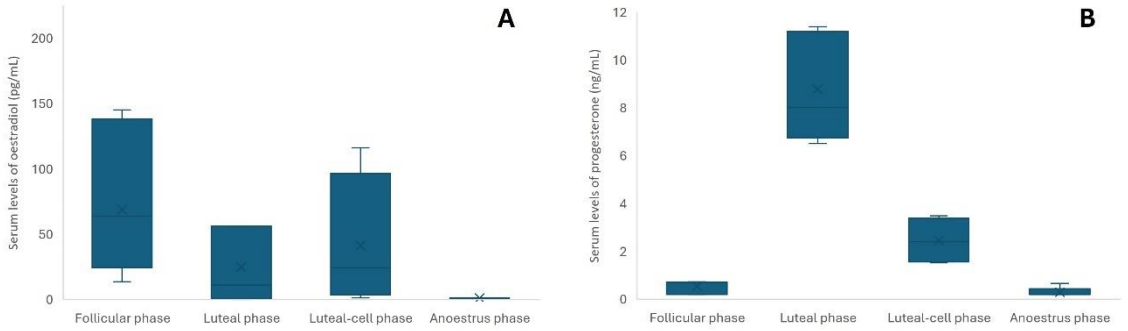

Figure 1. Serum hormonal concentration. A, serum concentration of oestradiol. B, serum concentration of progesterone. Follicular phase, n=7. Luteal phase, n=8. Luteal-cell phase, n=4. Anoestrus phase, n=5.

3.3 Cytology findings

When comparing the different groups, statistically significant ($P<0.05$) differences were observed only in the percentage of luteal, mesenchymal cells and mitotic figures (Table 4). The other parameters analysed showed no statistically significant difference.

In general terms, all samples showed a light pink background with scant cellular debris and stain material that was considered artefacts. Only one of the queens, belonging to the anestrous phase group, had abundant debris, although still adequate for diagnosis. Likewise, the background of ovarian FNA contained low to moderate numbers of small round and clear vacuoles consistent with lipid droplets. The presence of amorphous, pink and proteinaceous material consistent with extracellular matrix was also variable among slides, although no significant statistical differences were observed between groups (Figure 2).

Regarding cellularity, 12 out of 48 (25%) smears showed abundant cellularity, cellularity was found adequate in 26 (54.2%) smears and 10 (20.8%) smears showed scant cellularity.

The cell distribution was generally adequate, showing minimal or no cellular overlapping in 40 out of 48 smears (83.3%). A total of 6 (12.5%) preparations had the cells distributed in restricted areas with moderate cell overlapping, and 2 (4.16%) smears had overlapped cells or cells distributed only in clumps.

Cell preservation was variable, ranging from adequate, with less than 25% of ruptured cells in 12 (25%) preparations, to poor, with more than 50% of ruptured cells in 4 smears (8.3%). The remaining 32 samples (66.6%) showed moderately adequate cell preservation with 25% to 50% of broken cells.

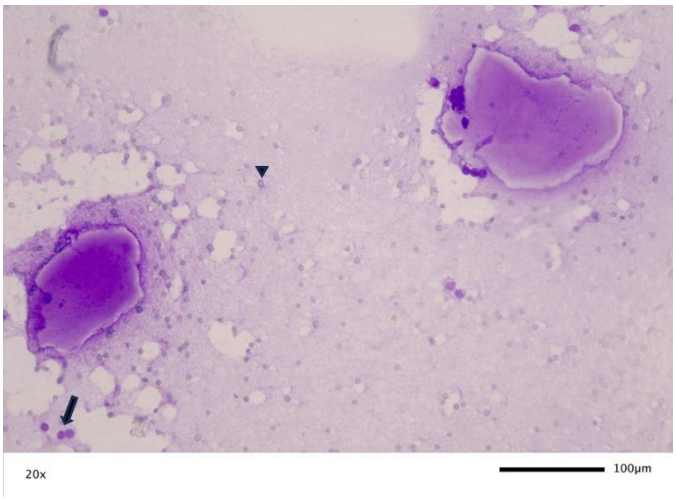

Figure 2: Cytology from a normal feline ovary obtained by fine needle aspiration. Modified Giemsa stain. Mild proteinaceous background with dense proteinaceous material (pink). Note the presence of erythrocytes (arrowhead) and granulosa cells (arrow).

Cellularity, cell distribution, cell preservation, fat droplets and proteinaceous material had non-significant statistical differences between groups.

The number of individual cells and cells distributed in clusters was generally similar, except in two queens from the luteal-cell group and one from the anestrous group, which contained higher numbers of clusters than individual cells, and two queens from the luteal-phase group, which contained higher numbers of individual cells compared with cells distributed in clusters.

Regarding the cell arrangement, different types of arrangement, such as acinar, palisade and papillary, were included and evaluated. No statistical differences were observed among the groups. The acinar arrangement was absent in 14 smears (29,2%), scattered in 24 (50%), few in 4 (8,3%) and moderate numbers in 6 smears (12,5%). Palisade arrangement was absent in 32 smears (66,7%), scattered in 14 (29,2%) and few in 2 smears (4,1%). Finally, the papillary arrangement was absent in 16 smears (33,3%), scattered in 12 (25%), few in 18 (37,5%) and moderate in 2 (4,2%).

Finally, the most common cell types identified on smears were granulosa, luteal and mesenchymal cells, and occasional leukocytes. Additionally, rare large multinucleated cells of unknown lineage were also found. Granulosa cells were distributed in different-sized aggregates, occasionally arranged in palisade or acinar-like structures. The cells were round to slightly cuboidal and small. Nuclei were round to slightly oval, centrally

or paracentrally located, with finely reticular chromatin and one or more small-sized nucleoli. The nuclear to cytoplasmic ratio was moderate to high. The cytoplasm was generally scant, clear blue and, sometimes, with low numbers of small clear vacuoles. Anisocytosis and anisokaryosis were mild (Figures 3A and 3B). There were no statistical differences in the number of granulosa cells among the evaluated groups.

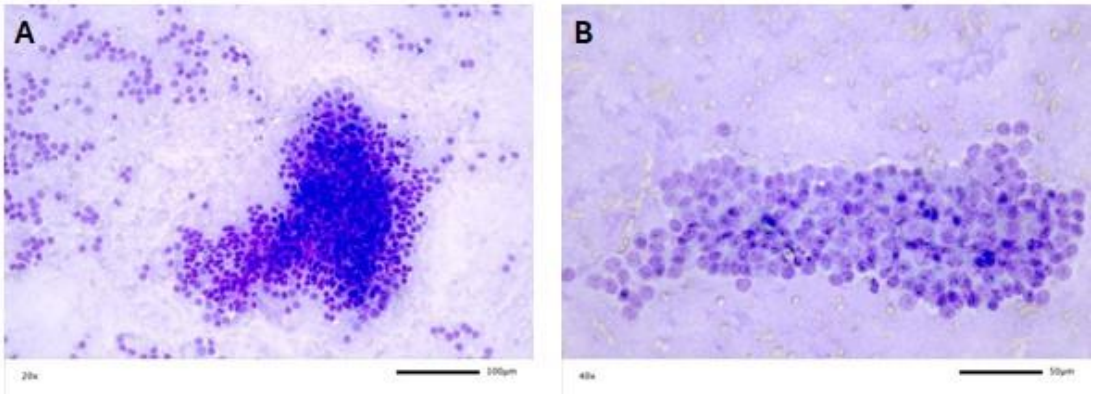

Figure 3: Fine needle aspiration of a normal ovary. Modified Giemsa stain. A: cluster of granulosa cells in a proteinaceous background. B: Papillary distribution of granulosa cells showing scant, clear blue cytoplasm with mild anisocytosis and anisokaryosis.

Luteal cells were large, round to slightly polygonal, generally individually arranged or rarely in loose aggregates. Nuclei were round, central or paracentrally located, with finely reticular chromatin and, occasionally, with one nucleolus visible. The nuclear:cytoplasmic ratio was low. The cytoplasm was generally abundant, with distinct cell borders, finely granular and blue to purple (Figure 4). Frequently, cells contained variable numbers of small to medium-sized clear vacuoles. Anisocytosis and anisokaryosis were mild to moderate, and scant binuclear cells were found. Luteal cells appeared in 71.4% of the preparations from the CL phase group, whereas they were absent in the other groups (Table 4). In this sense, categories 2 and 3 were observed in 28.6% of FNA each, whereas category 4 accounted for 14.3% of the samples.

Mesenchymal cells were fusiform, with thin wispy tails and were generally exfoliated in loose aggregates around a variable amount of amorphous pink material compatible with the extracellular matrix. The nuclei were oval, centrally placed and with finely reticular chromatin. The nuclear:cytoplasmic ratio was low to moderate. The cytoplasm was clear

blue and had well-defined cell borders. Anisocytosis and anisokaryosis were mild (Figure 5).

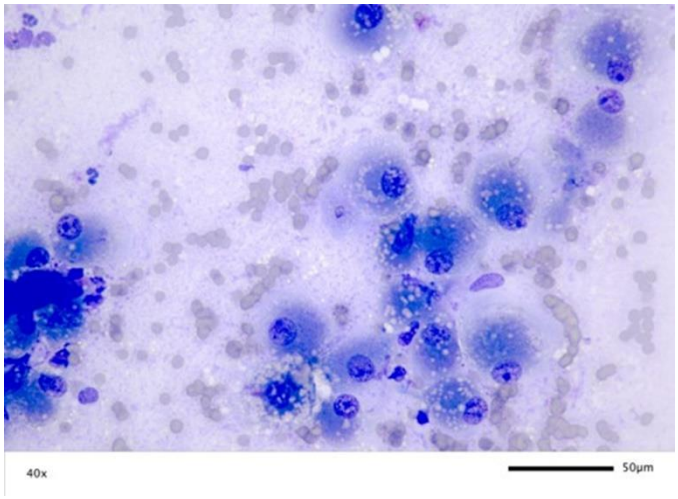

Figure 4: Fine needle aspiration of a normal ovary. Modified Giemsa stain. Luteal cells were characterised by clear blue vacuolised cytoplasm. The nuclei were eccentrically located.

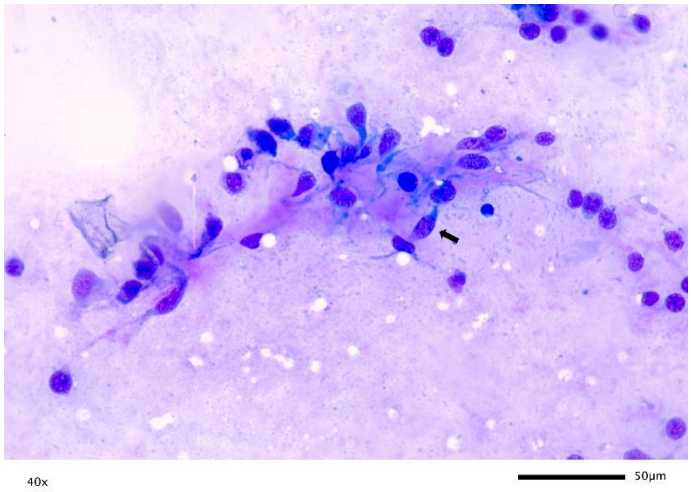

Figure 5: Fine needle aspiration of a normal ovary. Modified Giemsa stain. Occasional spindle cells with an oval nucleus and wispy cytoplasmic borders surrounding a small amount of pink matrix can be observed.

Table 4. Results of luteal and mesenchymal cells and mitotic figures at the different sexual phases evaluated in the study. Values are expressed in percentages (n). Statistically significant differences in the distribution of the cell types are represented with different superscripts.

	Category 0	Category 1	Category 2	Category 3	Category 4
Luteal cells					
Anoestrus (n=5) a	100 (n=5)	0	0	0	0
Follicular (n=7) a	100 (n=7)	0	0	0	0
Luteal phase (n=7) b	28.6 (n=2)	0	28.6 (n=2)	28.6 (n=2)	14.3 (n=1)
Initial luteal phase (n=5) a	100 (n=5)	0	0	0	0
Mesenchymal cells					
Anoestrus (n=5) a	60 (3)	0	40 (2)	0	0
Follicular (n=7) b	14.3 (1)	85.7 (6)	0	0	0
Luteal phase (n=7) c	42.9 (3)	28.6 (2)	28.6 (2)	0	0
Initial luteal phase (n=5) d	0	20 (1)	60 (3)	20 (1)	0
Mitosis					
Anoestrus (n=5) a	80 (4)	0	20 (1)	0	ND
Follicular (n=7) b	57.1 (4)	42.9 (3)	0	0	ND
Luteal phase (n=7) c	100 (7)	0	0	0	ND
Initial luteal phase (n=5) c	100 (5)	0	0	0	ND

Mesenchymal cells were present in 100% of samples from the luteal-cell phase, 85.7% in the follicular phase, 57.1% in the CL phase and 40% in the anoestrus phase (Table 4). Category distribution showed that all the samples from the anoestrus phase were classified as category 2. In the follicular phase, all the samples were classified as category 1. Regarding the CL phase, categories 1 and 2 accounted for 28.6% each. Finally, in the luteal-cell phase, categories 1 and 3 accounted for 20% of the samples each, whereas category 2 accounted for 60% of the samples. Queens belonging to the follicular group had significantly ($P<0.05$) lower numbers of mesenchymal cells.

Scant multinuclear large cells from unknown lineage, suggestive of being pycnotic granulosa cells, were also observed in the preparations from seven animals (Figure 6). Specifically, two animals from the anestrous phase group, one from the follicular phase group, two from the CL phase group and two from the luteal-cell phase. Cells were round to slightly polygonal and individually arranged. The nuclei were round, centrally located and with finely reticular chromatin. The nuclear to cytoplasmic ratio was low. Cytoplasm was generally abundant, finely granular and blue to purple. Anisocytosis and anisokaryosis were mild to moderate.

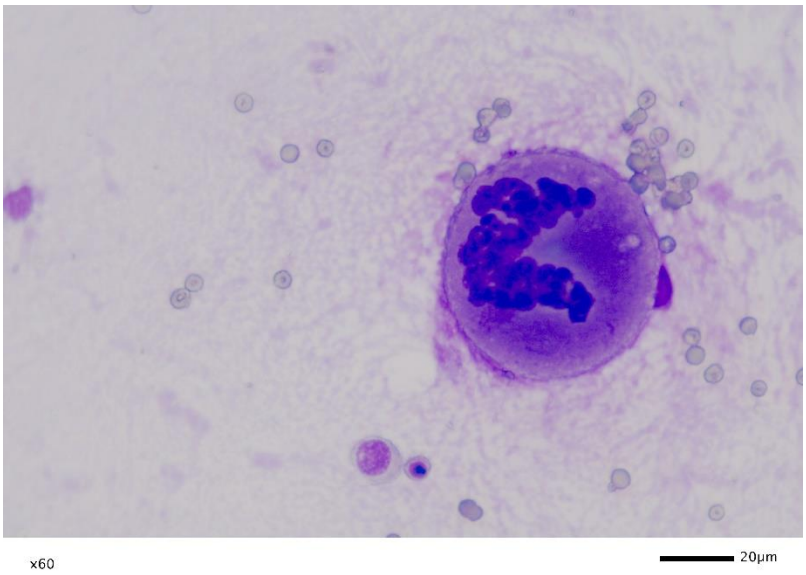

Figure 6: Fine needle aspiration of a normal ovary. Modified Giemsa stain. One single, large and multinucleated cell suggestive of being a pycnotic granulosa cell.

Mitotic figures also showed statistically significant differences among the groups (Table 4). Mitotic figures were present only in the follicular (42.9% of samples) and the anoestrus (20% of samples) phases. Noteworthy, in the anoestrus group, 20% of the animals showing mitotic figures were classified in score 2. In the follicular group, 57.1% of the queens had no mitotic figures, and 42.9% were classified as score 1, thus having scattered mitotic figures in the smears.

Leukocytes were present in 42 specimens, being present in all groups. A leukocyte score of 1 (1-3/400x) was present in 20 of the 42 positive smears. Of these, 12 had a blood contamination score of 1, 6 smears had a score of 2 and 2 smears had a score of 3. A leukocyte score of 2 (4-6/400x) was present in 12 smears. Of these, 6 had a blood contamination score of 2 and 6 had a score of 3. Finally, 10 smears showed a leukocyte score of 3 (7-10/400x). Of these, 2 had a blood contamination score of 2 and 8 had a blood contamination score of 3. In all the specimens with leukocytes, neutrophils predominate with a lower proportion of lymphocytes and eosinophils. Leukocyte presence was associated with the blood content in all the specimens. In four smears from the CL phase group, neutrophils were detected admixed with the luteal cells and sporadically in the cytoplasm, suggesting emperipolesis (Figure 7A and B).

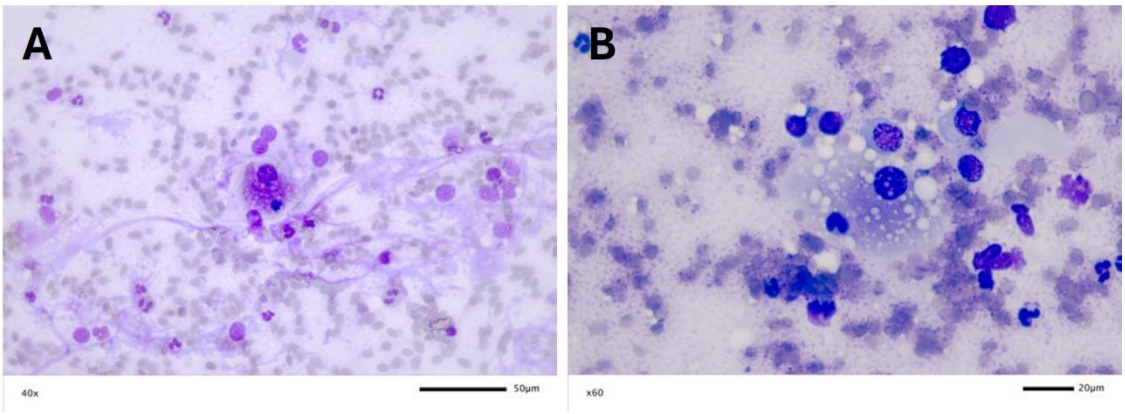

Figure 7: Fine needle aspiration of a normal ovary. Modified Giemsa stain. A: Mixed population of granulosa and luteal cells. In the center of the image, a luteal cell with an intact leukocyte in the cytoplasm can be observed (emperipolesis). B: Luteal and granulosa cells with naked nuclei in a background with red blood cells and leukocytes.

3.4 Histology findings

The presence of CL and ovarian follicles was evaluated on histological sections from the contralateral ovary in every queen (Figure 8). All the queens in the CL phase group (29.1% of the total population) had fully formed CL. Luteal cells were large, round to polyhedral and contained a round nucleus. The cytoplasm was moderately to heavily vacuolated. The remaining queens (70.8% of the population) did not present a defined CL. Twelve queens (50%), 4 from the anestrous phase, 5 from the follicular phase and 3 from the luteal-cell phase, had a mixture of large and small luteal cells, accompanied by ovarian follicles. The remaining five (20,1%) queens, 1 from the anestrous phase, 2 from the follicular phase and 1 from the luteal-cell phase, had no relevant numbers of luteal cells.

Finally, all the ovaries evaluated in the present study contained variable numbers of ovarian follicles regardless of the stage of the sexual cycle.

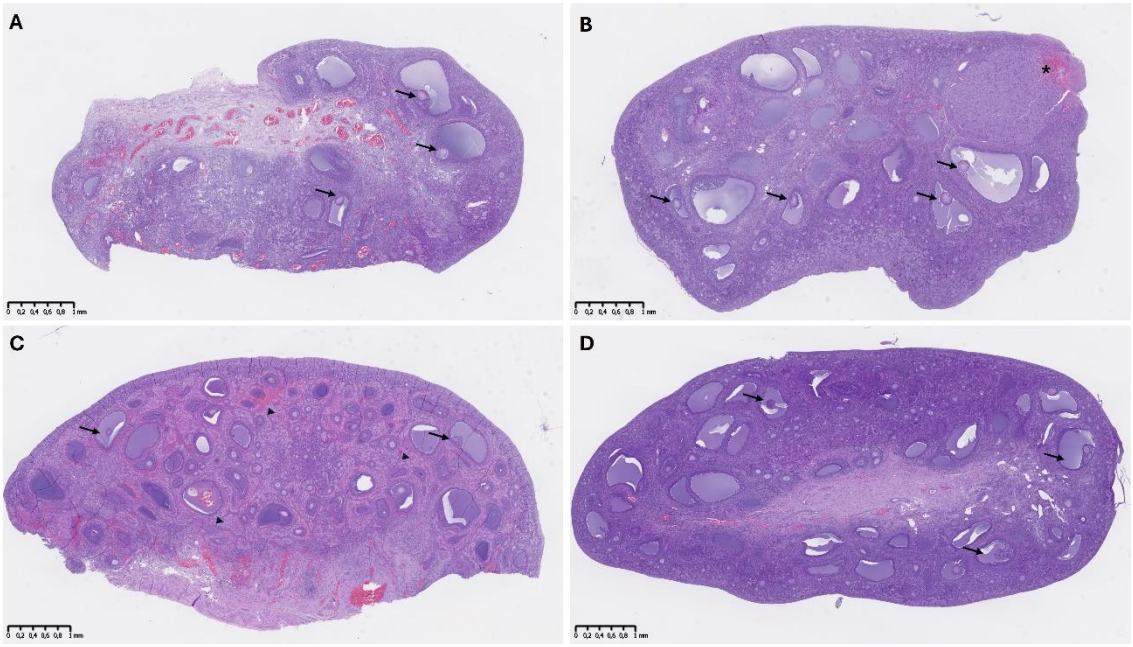

Figure 8: Ovarian histology. A: follicular group, queen number 1. Antral follicles with visible oocytes are observed (arrows); B: luteal group, queen number 15. A corpus luteum (*) and antral follicles with visible oocytes (arrows) can be observed; C: luteal cells group, queen number 16. Antral follicles with visible oocytes (arrows) and luteal cells not organised as a CL (arrowheads) are observed; D: anoestrus group, queen number 21. Antral follicles with visible oocytes are observed (arrows).

4. Discussion

In veterinary medicine, cytology has remarkably evolved in the last 15 years and is an extended diagnostic tool in general practice nowadays. Compared with histopathology, one of the main advantages of this diagnostic tool is obtaining rapid results with a less invasive and expensive technique (Marrinhas et al., 2022). When evaluating the reproductive system in small animals, cytology is more commonly used to assess mammary masses and to stage the oestrous cycle. However, little information on the physiological ovarian cytological features in dogs and cats is available (Solano-Gallego, 2023). On those species, ovarian cysts and tumours are the most commonly documented diseases diagnosed by cytology (Bertazzolo et al., 2004; Solano-Gallego, 2023), demonstrating that this tool has a good agreement (94.7%) with histology to diagnose canine ovarian pathologies.

According to the authors' knowledge, this is the first time that cytological features of feline ovaries throughout different sexual phases, sustained by histologic and hormonal findings, are described in the literature. In the present study, only the specific type of cells showed statistically significant differences among the studied groups. Thus, whereas granulosa cells were observed in all the sexual stages, luteal cells were present only in the cytology of those queens in the CL phase. This is an expected result since those were the only females showing an evident CL in ovarian histology. This finding agrees with that previously described by Piseddu et al. (2012) in dog ovaries.

Mesenchymal cells were observed in the ovarian cytology in all the groups. However, lower numbers were detected in the animals belonging to the follicular phase. The presence of mesenchymal cells in ovarian cytology has been previously described in the canine ovary (Piseddu et al., 2012; Solano-Gallego, 2023), although they were not associated with any specific oestrous stage. In the canine ovary, the exact origin of these cells was not determined, but they were classified as probably fibrocytes and fibroblasts. In addition, recent publications support the presence of ovarian-derived mesenchymal stem cells (MSCs) in the canine ovary (Trindade et al., 2017). Those cells appear morphologically as mesenchymal cells and express mesenchymal markers. The ovary is a constantly changing organ. Injury occurs during follicular rupture and ovulation, leading to a constant remodeling process. The presence of MSC in the ovary has been suggested to be involved in the tissue repair process after ovulation. Furthermore, these cells are suspected to contribute to forming granulosa cell-like epithelial cells (Trindade et al., 2017). Thus, the presence of mesenchymal cells in the feline ovary may also be associated with the reparation processes.

The mitotic index assessed in cytology also showed significant differences among the evaluated groups. Mitoses were only present in the follicular and anoestrus groups. The transformation of primordial follicles into secondary and, further, antral follicles involves the proliferation of the granulosa cells (Kosebent et al., 2018). In women's ovaries, the mitotic index of granulosa cells increases along with the diameter increase of the ovarian follicles (Gougeon, 1998), thus being related to folliculogenesis. In our samples, the exact origin of the cells undergoing mitosis was not determined, although, according to the cytological features, granulosa cells were suspected. However, other similar appearing cells, such as ovarian germinal cells, cannot be excluded (Piseddu et al., 2012). Likewise, in the present study, the highest percentage of mitotic figures was observed in the queens from the follicular group, making this result expected.

Large and multinucleated cells were also present in all the groups, with no association with any specific sexual stage. According to the authors' knowledge, the structures have not been previously described, and their exact origin has not been determined. However, the first hypothesis is that these cells correspond to pycnotic granulosa cells due to their large size and the presence of a multilobulated condensed nucleus. No correlation between these cells and the reproductive stage was observed.

Finally, variable numbers of leukocytes were present, predominantly neutrophils and lymphocytes, with few eosinophils. The presence of leukocytes was associated with blood contamination in most samples. However, in four smears from the CL group, a higher proportion of leukocytes were admixed with the luteal cells and, sporadically, in their cytoplasm, suggesting that leukocytes were truly present in the ovary. The presence of leukocytes in the cytoplasm of other cell types is known as emperipolesis. Luteal emperipolesis has been previously reported as a novel feature in normal canine ovaries (Piseddu et al., 2012) and other species, including humans, pigs and rats (Bukulmez and Arici, 2000; Oakley et al., 2011; Oakley et al., 2010).

Leukocytes play a crucial role in reproduction as they are considered potent *in situ* modulators of ovarian function. Their infiltration and distribution in the ovary are orchestrated by changes in reproductive hormones, such as steroids and gonadotropins, throughout the oestrous cycle. The main functions related to the presence of leukocytes in the ovary are the loosening of the follicular wall to facilitate follicular growth and ovulation, tissue repair after the follicle rupture and contribution to the luteal formation and regression (Bukulmez and Arici, 2000; Oakley et al., 2011).

Ovarian cytology may be a powerful technique to diagnose pathologies, and the knowledge of the specific physiological features is mandatory to distinguish a pathological statement. Cytology has been demonstrated to be a reliable tool to diagnose certain types of canine ovarian neoplasia. In cats, the information on ovarian cytology is scarce and is limited to neoplastic processes (Solano-Gallego, 2023). However, the small size of the queen ovaries impairs the sampling obtention, limiting its usefulness to cases where the organ is remarkably increased. In that scenario, and as similarly described in other species, it should also be taken into consideration that cytological investigations of ovarian masses may induce tumour spillage and its use should be limited to patients with evident metastatic disease or with a low body condition that does not allow the possibility of exploratory laparotomy (Bertazzolo et al., 2004). Another limitation of the present study is that it uses one ovary for cytological purposes and the contralateral one for

histological purposes. Since ovaries are synchronised structures, sampling both would provide a more reliable and robust diagnosis. However, the FNA technique may alter the ovarian structure and impair the histological assessment. Therefore, one of the ovaries was used for cytology purposes, and the other was used for histological evaluation. Lastly, in the present study, the groups were not evenly represented. Additionally, they included a low number of individuals.

5. Conclusions

The cytological description of healthy cat ovaries is a necessary preliminary step for the correct interpretation of the cytology of this organ. Ovarian cytology varies according to the reproductive cycle. Luteal cells are present in the corpus luteum (CL) phase. In contrast, mesenchymal cells are more common in the follicular and CL phases, and mitotic figures are found more often in the anoestrus and follicular phases.

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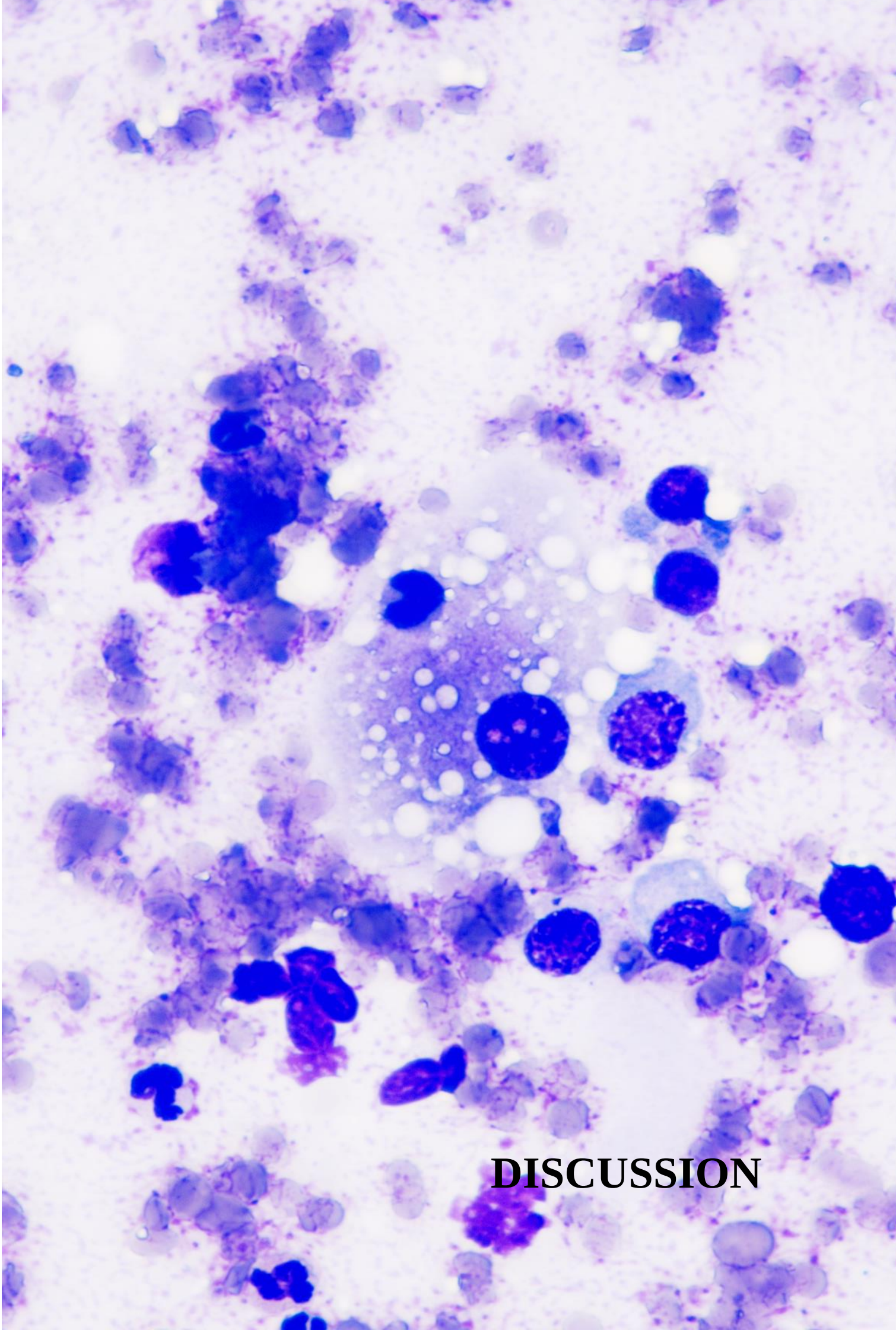


DISCUSSION

IX. DISCUSSION

Cats have become very popular family members worldwide and represent the most common pet in Europe (Eriksson et al, 2017), also being very common in the United States. This fact results in an increasing interest in their health and wellness. However, for many years, cats have been wrongly considered 'small dogs' and several fields, including reproduction, have been misinterpreted (Fontbonne, 2022). Currently, there is a lack of knowledge about feline reproduction and some causes of infertility are still poorly understood, causing important economic and genetic losses, especially in breeders of pedigree cats (Fontbonne et al., 2020; Prochowska & Nizanski, 2022). This void of information has increased the demand for this topic to be investigated, including the use of Assisted Reproductive Techniques (ARTs) currently used in other species like dogs (Prochowska & Nizanski, 2022).

Cytology is a commonly used technique in current veterinary medicine. Its popularity relies on the possibility of obtaining a rapid, minimally invasive and low-expensive diagnostic approach (Marrinhas et al., 2022), and it is applied in several tissue types, including the reproductive tract. In cats, the use of cytology in the reproductive tract is scarce compared to dogs, mares and cows (Kustritz, 2006; Cocchia et al., 2012; *Barański* et al., 2012; *Kasimanickam et al., 2005*). In bitches, cytology is frequently used to stage the oestrus cycle (Kustritz, 2012). In mares, the diagnosis of endometritis in subfertile females is based on endometrial cytology in conjunction with endometrial culture and/or biopsy (Agámez et al, 2021). In cows, subclinical endometritis is indicated by a polymorphonuclear neutrophil (PMN) threshold in cytological smears obtained from a cytobrush or uterine flushing (Wagener et al, 2017).

The primary aim of this PhD thesis was to assess the effectiveness of cytology in obtaining representative samples from the uterus of queens and to evaluate its potential in enhancing the diagnosis of fertility issues in this species. In the first article (MANUSCRIPT 1), the utility of cytology to investigate endometrial health was assessed by comparing two different habitual cytological techniques: uterine lavage (UL) and uterine swabbing (US). For this purpose, different parameters comprising the smear quality, the smear cellularity (including the characterisation of the endometrial cells), the

calculated cell scores, and the evaluation of inflammation focusing on the presence and disposition of PMN, were examined. Histopathology, bacteriology and serum levels of progesterone were used as controls to establish the status of the endometrium.

The results indicate that both sampling methods (UL and US) efficiently collected sufficient endometrial cells for diagnostic purposes. However, UL samples were more aligned with histological results than US samples. US samples exhibited significantly higher ratios of red blood cells (RBC)/endometrial cells and higher white blood cell (WBC) counts. These variations are likely attributed to the friction created by the swab against the endometrial surface during the sampling process, which may have caused the rupture of superficial blood vessels, with consequent bleeding. The bleeding probably contributed to the increase in WBC percentages and the higher RBC/endometrial cell ratios obtained.

In mares, although the uterine biopsy is, for many practitioners, still considered the more reliable technique for the diagnosis of endometrial health (Nielsen, 2005; Dlugolecka et al., 2019), the use of cytology is increasing its popularity and several studies have demonstrated that the use of endometrial cytology in combination with endometrial culture increases the accuracy to detect endometritis (Teixeira-Soares et al., 2022; Chedgey, 2022). In this species, three sampling techniques have been compared: guarded culture swab, uterine cytobrush, and low-volume uterine flush, with no significant difference in neutrophil recovery (Defontis et al., 2011). The cytobrush method has been found superior, yielding more diagnostic smears and correlating better with β-hemolytic streptococci cultures (Walter et al., 2012). This disagrees with what has been observed in the present study with queens. When comparing the two cytological techniques, the UL method was more representative than the US. Furthermore, an important consideration to bear in mind is that only UL is a clinically applicable technique in queens.

In the case of bitches, while cytology can provide insights into the uterine condition, studies have found low agreement between cytological and histopathological diagnoses of endometritis (Praderio et al., 2019; Bukowska et al., 2020), and biopsy is considered a reference method (Bukowska et al., 2020). Endometritis is a common subclinical uterine pathology, particularly in older bitches. Interestingly, subclinical endometritis is detected by histopathology in a high proportion of animals with good macroscopic uterine health and without clinical signs (Bukowska et al., 2020).

In the present study, hormonal status did not affect the cytology results. These results disagree with those observed in bitches and mares. Endometrial cytology in dogs varies throughout the reproductive cycle, with changes in cell types and morphology observed (Watts et al., 1998; Groppetti et al., 2010). In mares, the stage of the reproductive cycle may influence cytological results, with better agreement between histology and cytology during estrus (Kozdrowski et al., 2015).

Histologically, the results confirm the previously reported data on the increase in endometrial epithelium height. To the authors' knowledge, this is the first study to include histological evaluations of glandular density and diameter to better understand the histological characteristics of queens throughout different phases of their reproductive cycle. Our results align with previous research conducted on mares, particularly on day 7 of pregnancy (Camozzato et al., 2019). Specifically, increased glandular diameter, height of the glandular epithelium, and glandular lumen were observed in 7-day-pregnant mares in comparison with cyclic mares. These changes are significant as they coincide with the embryo's entry into the uterus. Notably, the increased height of the luminal epithelium and glandular diameter may be linked to the production of glandular secretions, which are released into the uterine lumen during early pregnancy.

The results from this study suggest that cytology may be a useful diagnostic tool for assessing endometrial status and, in turn, in the diagnosis of subfertility and infertility in queens. The small size of the uterus in this species makes it challenging to obtain a representative biopsy sample, resulting in limitations in diagnosing endometritis and consequently in detecting infertility problems. While histology is the prevalent method for diagnosing endometritis in many species, cytology could be an alternative technique for assessing the endometrium in smaller species like cats.

An important limitation of this study is the lack of queens with endometritis. Female cats are usually neutered at a young age, thus impeding the collection of samples from old queens. Therefore, further research is warranted to fully elucidate the utility of endometrial cytology in queens undergoing either clinical or subclinical endometritis.

The usefulness of cytology as a tool to evaluate ovarian health in queens was assessed in the second study (MANUSCRIPT 2). Understanding the ovarian-specific cytological characteristics is necessary for the detection of pathological diseases. However, to the

best of the author's knowledge, the characterisation of the ovarian features in queens remains limited and existing information primarily relies on histopathological (Amelkina et al., 2015) and ultrasound studies (Vercellini et al., 2021; Vercellini et al., 2018; Gatel et al., 2016) with cytological data mostly restricted to a few reports on neoplastic conditions (Solano-Gallego, 2023).

This study aimed to characterise the cytological features of the healthy queen ovary through the different stages of the oestrous cycle, supported by histological and hormonal findings. Different parameters were evaluated, including the quality of the smears, cellularity and cell preservation, the specific type of cells encountered, the presence of mitotic figures, the blood content and the number and distribution of the leukocytes. The histological features and the serum levels of oestradiol and progesterone were used to classify the queens into 4 groups: the anestrous, the follicular, the corpus luteum (CL) and the luteal-cell groups. No statistically significant differences were observed in the evaluated parameters among groups, except for the percentage of luteal and mesenchymal cells and mitotic figures.

As expected, granulosa cells were observed in cytological samples from all the different sexual stages, whereas luteal cells were only present in those queens from the CL phase. These findings agree with those previously documented by Piseddu et al. (2012) in canine ovary cytology.

In contrast with the previously described, our study showed lower amounts of mesenchymal cells in the queens belonging to the follicular phase. In the canine ovary, mesenchymal cells were present in all the oestrous stages and were not associated with any specific oestrous stage. It has been proposed that mesenchymal stem cells (MSC) play a role in the tissue repair mechanisms following ovulation in the canine ovary (Trindade et al., 2017). Additionally, these cells are believed to contribute to the formation of granulosa cell-like epithelial cells (Trindade et al., 2017). Therefore, the presence of mesenchymal cells in the ovaries of felines may also be linked to repair processes.

Mitoses were observed exclusively in the follicular and anoestrus groups. Although the exact type of the cells undergoing mitosis was not determined, granulosa cells were suspected according to the cytological features. In women's ovaries, the mitotic index

of granulosa cells rises as the diameter of the ovarian follicles increases, establishing a connection to folliculogenesis (Gougeon, 1998). In our study, the highest percentage of mitotic figures was observed in the queens from the follicular group, making this result expected.

As a new unexpected finding, large multinucleated cells were observed in all the groups, and there was no association with any specific sexual stage. To the best of the author's knowledge, these cells have not been previously documented. Their precise type remains unclear. However, their considerable size suggests that they may be apoptotic granulosa cells.

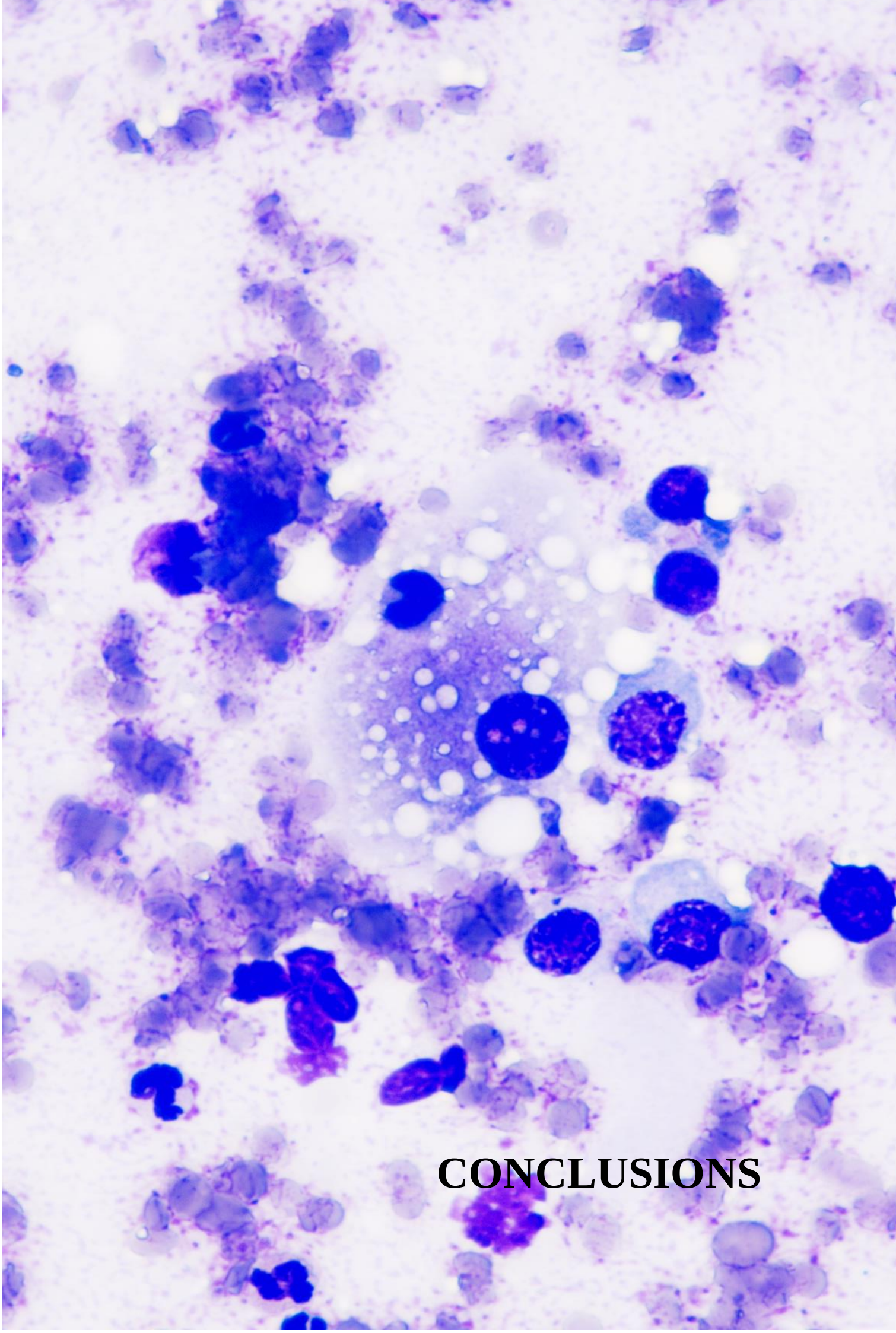
Finally, the number and proportion of leukocytes present in the samples were generally associated with the blood content of the smears. However, in some of the animals belonging to the CL group, a higher proportion of leukocytes were present and occasionally, luteal emperipolesis was detected. Luteal emperipolesis was previously described as a novel feature in normal canine ovaries (Piseddu et al., 2012). Emperipolesis is a type of cell engulfment in which an intact and viable hematopoietic cell is seen in the cytoplasm of a host cell (Gupta et al., 2017). This phenomenon is present in different physiological and pathologic conditions and several cells have been described as host cells, including megakaryocytes, monocytes, endothelial cells, fibroblasts and malignant cells (Gupta et al., 2017).

Leukocytes have a relevant role in the *in situ* modulation of ovarian function. Their functions include loosening the follicular wall to facilitate follicular growth and ovulation, tissue repair after follicle rupture, and contribution to luteal formation and regression (Bukulmez and Arici, 2000; Oakley et al., 2010).

Little is known about queens' ovarian cytology, and this article provides the basis for future studies. More is known about the bitch, where the cytological evaluation of canine ovarian tumours and normal ovaries have provided valuable diagnostic information. Normal ovarian cytology varies with the oestrous cycle, with luteal cells prominent in diestrus and showing characteristic changes from early to late diestrus (Piseddu et al., 2012). Some ovarian pathologies, like ovarian carcinomas, typically present with abdominal effusions containing cells arranged in papillary patterns, showing high diagnostic accuracy (Bertazzolo et al., 2012). Also, various ovarian

tumours, including papillary adenocarcinomas, granulosa cell tumours, and teratomas, can be identified through distinct cytological features (Bertazzolo et al., 2004). In cats, cytological examination of fine needle aspirates can be valuable in identifying ovarian masses, as demonstrated in a case of ovarian luteoma, which presented large round to oval cells with basophilic cytoplasm and vacuoles (Choi et al., 2005).

Further investigations are necessary to develop the usefulness of this technique as a powerful diagnostic tool in the routine clinic.



CONCLUSIONS

X. CONCLUSIONS

1. Endometrial cytology, combined with uterine culture, is helpful to assess the endometrial health of the queen, where obtaining histological specimens is challenging.

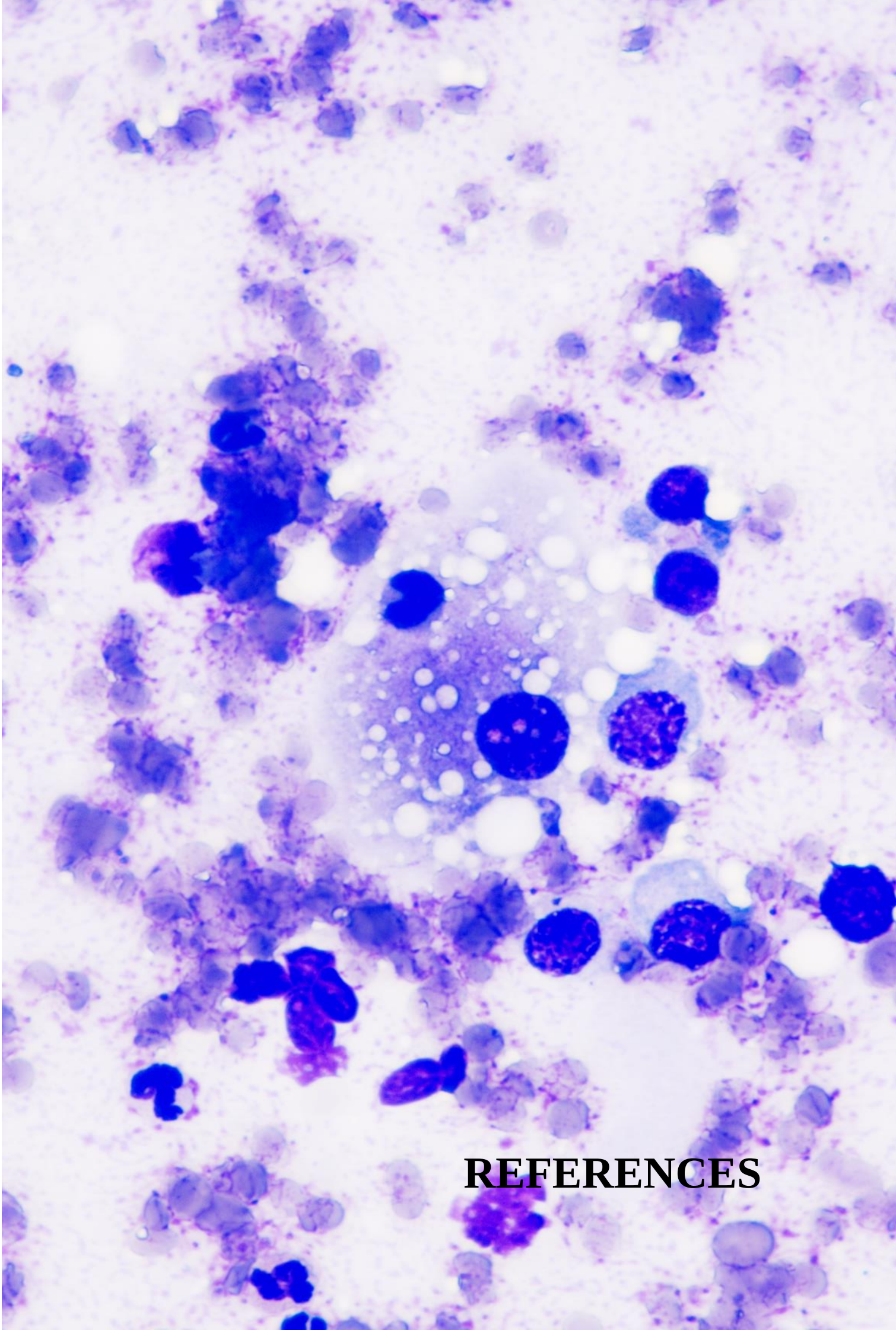
2. Both uterine brushing and uterine lavage techniques allow the cytological examination of endometrial samples in queens.

3. The uterine lavage technique is in close alignment with histological results.

4. Ovarian cytology allows obtaining samples of good quality comparable to histology assessment.

5. Ovarian cytology varies according to the reproductive cycle.

6. Endometrial, either by brushing or lavage technique, and ovarian cytology are promising new, helpful tools to assess the reproductive tract in the queen.



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