

Distribution of Plasma Cells and Alpha-Naphthyl Acetate Esterase - Positive Lymphocytes in the Reproductive Organs of Female Cats in Anoestrus, Oestrus and Pseudopregnancy

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SUMMARY

The distribution of plasma cells and alpha naphthyl acetate esterase (ANAE) positive lymphocytes in the reproductive tract and ovaries of cats in anoestrus, oestrus and pseudopregnancy was determined using histochemical methods. For plasma cell determination, tissue samples were fixed in formol-alcohol for 48 hours, embedded in paraffin and six-micrometer-thick sections stained with methyl green-pyronine. For ANAE demonstration, tissue samples were fixed in formol-sucrose fixative (pH 6.8), stored for 22 hours at +4 °C and then additionally fixed in Holt's solution for 22 hours at +4 °C. Cryostat sections of 8 µm thickness were stained for ANAE activity at pH 6.2. The number of plasma cells was significantly lower (P<0.05) in the uterine corpus than the uterine horn of anoestrus cats. Plasma cell numbers were significantly higher (P<0.05) in the ovarian cortex and medulla of the pseudopregnancy group than in the anoestrus and oestrus groups. The density of ANAE-positive lymphocytes was significantly (P<0.05) greater in the uterus and ovaries of oestrous and pseudopregnancy cats than anoestrous cats. It was concluded that the stage of oestrous cycle and pseudopregnancy could be responsible for changing the distribution of plasma cells and ANAE-positive lymphocytes in reproductive tract tissues and ovaries of female cats.

Key Words

Plasma cell, Lymphocyte, Oestrous cycle, Pseudopregnancy, Cat

Östrüs, Anöstrüs ve Yalancı Gebelikteki Kedilerin Dişi Üreme Organlarında Plazma Hücreleri ve Alpha-Naphthyl Acetate Esterase Pozitif Lenfositlerin Dağılımı

ÖZET

Kedilerde anöstrüs, östrüs ve yalancı gebelik dönemlerinde dişi genital kanal organları ve ovaryumlarında, plazma hücresi ve alpha naphthyl acetate esterase (ANAE) pozitif lenfositlerin dağılımı, histokimyasal teknikler kullanılarak ışık mikroskopik olarak belirlendi. Plazma hücrelerinin incelenmesi için doku örnekleri formol-alkol solüsyonunda 48 saat tespit edilip parafinde bloklandı. Altı mikron kalınlığındaki kesitler metil green - pironin ile boyandı. ANAE için doku örnekleri formol-sükroz solüsyonunda (pH 6.8) +4°C 'de 22 saat, sonra Holt solüsyonunda yine aynı şartlarda 22 saat fikse edildi. ANAE boyaması için 8 µm kalınlığındaki kriyostat kesitleri pH 6.2'de inkübe edildi. Kedilerde anöstrüs döneminde plazma hücre sayısı korpus uteride kornu uteriye göre anlamlı derecede azdı (P<0.05). Yalancı gebelik grubundaki kedilerin ovaryum korteks ve medullasında, plazma hücre sayısının, anöstrüs ve östrüs gruplarına göre daha yüksek olduğu belirlendi (P<0.05). ANAE-pozitif lenfositlerin yoğunluğu, östrüs ve yalancı gebelik dönemi kedilerin uterus ve ovaryumlarında anöstrüs grubu kedilerden anlamlı derecede yüksekti (P<0.05). Sonuçta, östrüs siklusu ve yalancı gebeliğin dişi kedilerde üreme kanal organları ile ovaryumlarda plazma hücreleri ve ANAE-pozitif lenfositlerin dağılımındaki değişimlerden sorumlu olabileceği kanısına varıldı.

Anahtar Kelimeler

Kelimeler: Plazma hücresi, Lenfosit, Östrüs siklusu, Yalancı gebelik, Kedi

INTRODUCTION

During early pregnancy, the endometrium creates an appropriate environment for the embryo, e.g. providing uterine secretions during early development (Dantzer 1985). During early and mid-pregnancy, leukocyte infiltration of the endometrium has been observed (Bischof et al. 1995; Karaca et al. 2009) and variations in

infiltration of intraepithelial lymphocytes during days 10-20 of pregnancy have been reported (King 1988). However, successful pregnancy in pigs is associated with intrauterine immunosuppression, suppression of major histocompatibility complex molecules and T cell responses, which allow the embryos to develop inside the uterus without being rejected (Croy et al. 1987; Kaeoket et

al. 2003). The endometrium is one of the most complex tissues, undergoing dynamic changes because it has to remodel in response to implantation and pregnancy processes. The implantation mechanism remains unclear because of various factors inherent in that process, such as cytokines, growth factors, hormones and adhesion molecules (Sharkey 1998; Salamonsen 1999).

Immune competent cells are present in the female reproductive tract (Hunt 1994), and human endometrium is an active site of cytokine production (Tabibzadeh 1991). Immunoglobulin secretion by the reproductive tract has been demonstrated in many species (Wira et al. 1994; Parr and Parr 1994). The major class of lymphocytes is the T cells, which can be divided into subpopulations of helper (TH) cells and cytotoxic (TC) cells (Roitt et al. 1998). In response to antigenic stimulation, TH cells release cytokines which promote the proliferation and/or differentiation of other T cells, B cells, natural killer cells and monocytes/macrophages (Kaeoket et al. 2003). Plasma cells are developed from B lymphocytes, in response to antigen. B cells are found in the lymph nodes and the spleen. The plasma cell progenitors migrate into the tissues and respond to antigen stimulation by division and differentiation into tissue plasma cells (Tizard 1996).

Nonspecific esterase is widely distributed in various types of cells. Cytochemical esterase activity is commonly used to differentiate types of leukocytes and leukemia cells (Maiti et al. 1990). Alphanaphthyl acetate esterase is a nonspecific esterase. The pattern of esterase activity revealed by this method provides a discriminating marker for mature T lymphocytes showing dense, localized, dot-like positive responses (Maiti et al. 1990; Cemek et al. 2006; Enginar et al. 2007).

Adult cats are described as seasonally polyoestrus and induced ovulators (Tsutsui and Stabenfeldt 1993; Arikan et al. 2009). If ovulated oocytes are not fertilized or pregnancy fails for other reasons, the female cat undergoes pseudopregnancy, which lasts approximately 40 days (Arikan et al. 2008; Paape et al. 1975).

The objective of this study was to evaluate the pattern of changes in plasma cell numbers in the uterus, uterine tubes and ovaries of cats in anoestrus, oestrus and pseudopregnancy and to assess the quantitative distribution ANAE positive lymphocytes in these stages.

MATERIALS and METHODS

Animals and Tissue Collection

Fifteen female cats presented for ovariohysterectomy to Faculty of Veterinary Medicine clinic at Kirikkale University were included in the study and divided into three groups, each containing 5 cats. One group of cats (Group I, anoestrus) was ovariohysterectomized in the anoestrus reproductive stage in off-breeding season, while the remaining 10 animals were ovariohysterectomized in the breeding season. Half of those animals were ovariohysterectomized on day 3 of oestrus (Group II, oestrus) and the remaining group (Group III, pseudopregnancy) on day 10 of pseudopregnancy in which ovulation was induced by intensive vaginal stimulation with a glass thermometer. All animals were free from genital diseases. The experimental protocol used was approved by the University of Kirikkale Ethical Committee, and adhered to the European Community Guiding Principles for the Care and Use of Animals.

Reproductive organs were transported to the laboratory immediately after surgery. Tissue samples were collected from the uterus (horn and corpus), fallopian tubes (isthmus and ampulla) and ovaries including corpora lutea pseudopregnant animals.

Histological procedure

Each tissue sample was cut into two equally-sized pieces. One set of sub-samples was fixed for 48 hours (h) in formol-alcohol (for plasma cells) and cross-sections were processed routinely for light microscopy by the paraffin technique. Sections of 6 μ m thickness were stained with methyl green-pyronine for evaluation of plasma cell distribution. Plasma cells were identified by the pink staining and the eccentrically placed and round nucleus by methyl green-pyronine. These tissue sections were examined under light microscopy (x200) and the number of plasma cells counted in random high-power fields using a Nikon Optiphot II light microscope incorporating a square graticule in the eyepiece. Ten serial sections were examined by light microscopy on the preparations obtained from each animal.

For ANAE demonstration (Aştı et al. 1997), the second set of sub-samples from each anatomical region was fixed in formol-sucrose solution (pH 6.8) for 22 h at +4°C. Following the first fixation, the samples were additionally fixed in Holt's solution for 22 hours at +4 °C. Cryostat sections 8-10 μ m thick were cut and determined for alpha naphthyl acetate esterase (Sigma, Germany) activity at pH 6.4 for 10 min. The incubated preparations were then washed in distilled water and counterstained with 1% methyl green for 30 min. Following dehydration in increasing concentrations of ethanol, the preparations were cleaned in xylene and mounted in DPX. After applying ANAE enzyme stain, the tissues were examined under a light microscope. The regional distribution of ANAE positive lymphocytes was compared in five anatomically distinct tissues of cats in anoestrus, oestrus and pseudopregnancy. The ANAE positive lymphocyte population in different tissues were estimated subjectively, and each tissue was awarded arbitrary score of 1 to 4 points according to whether it was considered to have exceptionally large numbers (++++), large numbers (+++), moderate number (++) or rare (+) ANAE positive lymphocytes (Karaca et al. 2007). Ten microscopic fields in five sections (8 μ m thickness) of each anatomical zone were evaluated using a x40 objective.

Statistical analysis

The data are expressed as mean \pm standard deviation (SD). Significant differences between the groups were determined with SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) for Windows using one-way analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test. Differences were considered significant at $P < 0.05$.

RESULTS

The endometrium contained greater numbers of plasma cells than the myometrium in all three groups of cats. The plasma cell counts for the uterus (horn and corpus), uterine tubes (isthmus and ampulla) and ovary are summarised in Table 1. The number of plasma cells in the uterine corpus region was significantly lower ($P < 0.05$) than in the horn of uterus in the anoestrus cats. Plasma cells numbers were higher found in the ampulla of oviduct than in the isthmus of oviduct. Plasma cells numbers were significantly ($P < 0.05$) higher in the ovarian cortex and

medulla of pseudopregnancy cats than in anoestrus and oestrus cats (Figures 1, 2, 3).

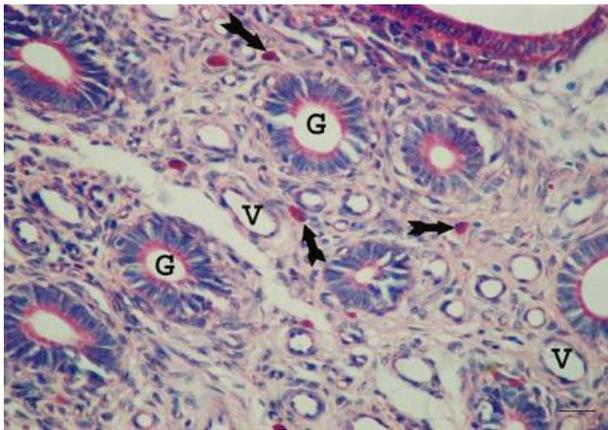


Figure 1. Plasma cells in the endometrium (Oestrus group). Arrows: Plasma cells; G: Uterine gland; V: Vessel. Methyl green-pyronine (Bar=60µm)

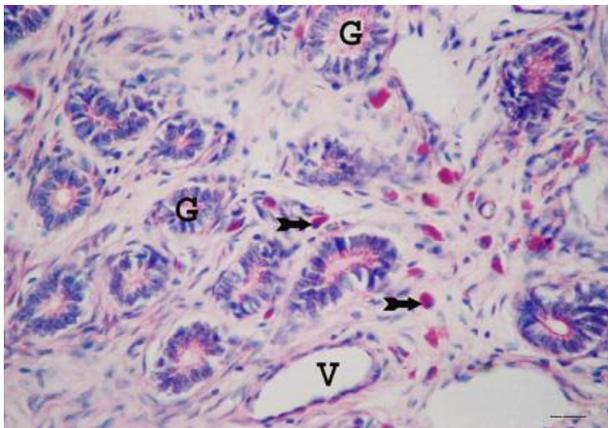


Figure 2. Plasma cells in the uterine horn (Pseudopregnancy group). Arrows: Plasma cells; G: Uterine gland; V: Vessel. Methyl green-pyronine (Bar=60µm)

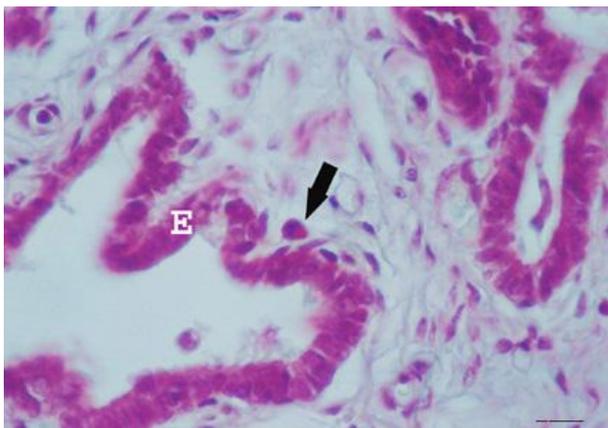


Figure 3. Plasma cell in the ampulla of oviduct (Anoestrus group). Arrow: Plasma cell; E: Epithelium. Methyl green-pyronine (Bar=40µm)

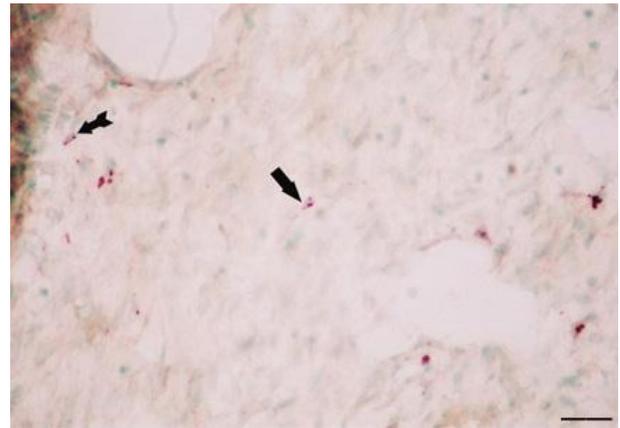


Figure 4. Alpha naphthyl acetate esterase (ANAE) staining (Pseudopregnancy group). Arrows: ANAE positive lymphocytes, ANAE, (Bar=60µm)

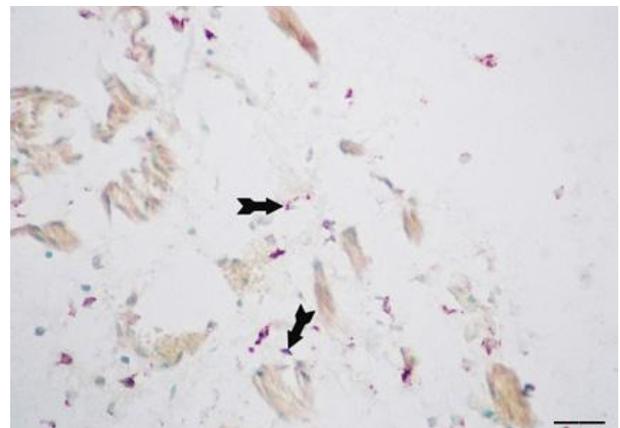


Figure 5. Alpha-naphthyl acetate esterase (ANAE) staining (Oestrus group). Arrows: ANAE- positive lymphocytes, ANAE, (Bar=60µm)

ANAE positive lymphocytes

ANAE positive lymphocytes were observed throughout the reproductive tract and ovaries at anoestrus, day 3 of oestrus and pseudopregnancy. The density of ANAE positive lymphocytes was markedly greater in the uterine horn and uterine corpus, day 3 of oestrus and pseudopregnant cats than in that of anoestrus cats. The density of ANAE positive lymphocytes were lower in oviduct than uterus and ovaries, and were also lower in pseudopregnant cats than anoestrus and estrus cats. The distribution of ANAE-positive lymphocytes that in the ovarian cortex and medulla was not influenced by stage of oestrus cycle (anoestrus and oestrus) (Figures 4, 5 and Table 2).

Table 1. Plasma cell number in female cat reproductive organs after staining with methyl green-pyronine (n = 5 for each group)

Group	Uterus		Oviduct		Ovary	
	Corpus	Horn	Ampulla	Isthmus	Cortex	Medulla
Anoestrus	56.1±12.6 ^b	54.7±9.4 ^b	47.4±6.1 ^b	39.7±6.4	51.9±9.1 ^b	52.4±6.7 ^b
Oestrus	69.5±11.0 ^a	65.3±8.3 ^a	56.5±11.3 ^a	41.4±20.1	56.8±6.4 ^b	53.8±9.8 ^b
Pseudopregnancy	71.8±24.4 ^a	69.9±11.6 ^a	60.4±8.4 ^a	42.0±14.6	67.4±11.7 ^a	57.2±8.5 ^a

Means within columns followed by different letters are significantly different (P<0.05)

Table 2. Relative occurrence of ANAE-positive lymphocytes in cat female reproductive organs

Group	Uterus		Oviduct		Ovary	
	Corpus	Horn	Ampulla	Isthmus	Cortex	Medulla
Anoestrus	++	+	+	+	+	+
Oestrus	+++	++	+	+	++	++
Pseudopregnancy	+++	+++	+	+	++	+

Explanations: (+) - few; (++) - moderate number; (+++) - large numbers; (++++) - exceptionally large numbers of ANAE positive lymphocytes; n = 5 for each group

DISCUSSION and CONCLUSION

Plasma cells and lymphocytes consistently comprised by far the greatest proportion of leukocytes within the reproductive tract organs and the ovarian tissue as a whole, and marked temporal fluctuations in the numbers of these cells were observed during the oestrus cycle and early pregnancy (Brannstrom et al. 1994). Brannstrom et al. (1994) reported neutrophilic granulocytes and monocytes/macrophages to be the most abundant leukocyte populations in the corpus luteum, during both pregnancy and pseudopregnancy, but found no significant changes in OX52-positive T-lymphocytes in the corpus luteum during either pregnancy or pseudopregnancy (Brannstrom et al. 1994).

In the present study, variations in the distribution of plasma cells were observed between the different regions of the reproductive tract and within these regions during cyclical changes and pseudopregnancy. The anoestrus uterus, oviduct and ovaries contained fewer plasma cells than those in oestrus and pseudopregnancy. This might suggest that plasma cells are specifically sensitive to hormonal alterations in cats.

In the present study ANAE-positive lymphocytes were not found in the muscular layer of the oviduct but were observed in the lamina propria, which is in agreement with earlier reports on goats (Karaca et al. 2007).

Mucosal intraepithelial T-lymphocytes are involved in immunosurveillance. For reproductive mucosa, it has been suggested that CD8⁺ intraepithelial cells may represent a population of suppressor cells that down regulate immunological reactions directed against spermatozoa, which carry autoantigens. Tekin and Hansen (2004) suggest that accumulation of macrophages during pregnancy is limited spatially to the stromal compartment of the endometrium. In the present study, ANAE-positive lymphocytes were present in significantly higher intense in corpus, horn and cervix of uterus in oestrus and pseudopregnancy cats than that in anoestrus cats. However, there were no differences in the density of ANAE-positive lymphocytes in uterine tubes of the pseudopregnancy group compared with the other groups. ANAE-positive lymphocytes were found in moderate to high density in the oviduct mucosa of cats.

It has been reported that CD8⁺ lymphocytes are localised between the epithelial cells of the uterine mucosa and endometrial glands in goats (Martinez et al. 2005). The CD8⁺ T-lymphocyte subpopulation is reported to be largest in the endometrial epithelium, glandular epithelium and stroma (Perez-Martinez et al. 2002). A previous study reported that CD4⁺ lymphocytes are abundant and widely distributed in normal feline female reproductive tract and that CD8⁺ cells are the most prevalent immune cells in the feline female reproductive tract (Butterworth et al. 2001).

In the present study we confirmed that ANAE-positive lymphocytes increase in the reproductive tract organs of

female cats. Our enzyme histochemical evidence confirms that ANAE-positive lymphocytes enhance the previously reported cellular changes related to the oestrous cycle (Karaca et al. 2007), and that these changes are similar to those observed in early pregnancy (Martinez et al. 2005). In the goat caruncula, all the lymphocyte subpopulation disappears from the placentome during gestation, while the number of CD4⁺ and CD8⁺ T-lymphocytes decreases drastically in the intercaruncular area (Martinez et al. 2005).

According to the data obtained in this study, the distribution of plasma cells and ANAE-positive lymphocytes can vary due to changes during the oestrous cycle and pseudopregnancy in the female reproductive organs of cats. Additional studies are needed to clarify the distributional mechanism of immune system cells in relation to the stage of the oestrous cycle and pseudopregnancy in cats.

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