Histological and Immunological Changes in *Uterus* During the Different Reproductive Stages at Californian Rabbit (*Oryctolagus cuniculus*)

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Abstract

Rabbit is the third most commonly used animal model in different fields of scientific research, such as reproductive biology, fertility and embryo transfer studies, and immunology. This animal species, often used in antibodies production, has minority of scientific records about the immunological status of its reproductive organs. The aim of this study was to find histological and immunological changes in rabbit female reproductive tract during different reproductive stages. The study was carried out on female rabbits, divided in three groups, according to the following stages of reproductive cycle: Estrous, ovulation and pregnancy. Histological and immunohistochemical stains for T- and B-cells were performed on tissue samples of cornu uteri and cervix. T lymphocytes were predominant in all anatomical parts of the uterus, in all stages of the cycle. The highest number of those cells was recorded at estrous, while the lowest was recorded at pregnancy. Cervix expressed more immunological activity than cornu uteri. The distribution and the number of immune positive cells in the rabbit female reproductive tract depend on its hormonal status.

Keywords: Rabbit, Uterus, Cervix, B-cell, T-cells

Kaliforniya Tavşanının *(Oryctolagus cuniculus)* Farklı Üreme Dönemlerinde Uterustaki Histolojik ve İmmunolojik Değişiklikler

Özet

Tavşan; üreme biyolojisi, üretkenlik ve embriyo transferi çalışmaları ve immünoloji gibi farklı alanlardaki bilimsel çalışmalarda en sıklıkla kullanılan hayvan modelleri arasında üçüncü sırada yer almaktadır. Antikor üretiminde kullanılan bu hayvanların üreme organlarının immünolojik durumu hakkındaki raporlar oldukça kısıtlıdır. Bu çalışmanın amacı farklı üreme devrelerinde bulunan dişi tavşanların üreme organlarında şekillenen histolojik ve immünolojik değişiklikleri belirlemektir. Dişi tavşanlar östrus, ovulasyon ve gebelik olmak üzere üç gruba ayrıldı. Cornu uteri ve serviks dokularında histolojik incelemeler ile T ve B-hücreleri için immunohistokimyasal boyamalar yapıldı. T lenfositler uterusun tüm anatomik bölümlerinde ve tüm dönemlerde baskın hücre olarak belirlendi. Bu hücreler en yüksek oranda östrusta ve en düşük gebelikte tespit edildi. Servikste cornu uteriden daha fazla immünolojik aktivite belirlendi. Immun pozitif hücrelerin dağılım ve sayısının dişi tavşanların üreme organlarında hormon durumu ile ilişkili olduğu sonucuna varıldı.

Anahtar sözcükler: Tavşan, Uterus, Serviks, B-hücre, T-hücre

INTRODUCTION

Although the rabbit is one of the most commonly used models for scientific researches in fundamental sciences and medicine [1], more immunological studies about local immune system in the female reproductive tract (FRT) have been taken on the rodent model. Even though the lack of available reagents for characterization of immune cells in the rabbit FRT could be the reason for choosing rodents for investigation, the similarity of Order Lagomorpha with

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humans has often been the crucial factor for using the rabbit model. This similarity is most easily distinguishable in very similar gene sequences [2] and in embryo and fetal differentiation. Limited information about changes in local immunity of the rabbit FRT during different reproductive stages was another reason to carry out this investigation using the rabbit model. Rabbit possesses *uterus duplex,* with two separate *cervices* and two *canalis cervicis uteri* in each *cervix* [3,4] with elongated uterine horns [5]. The topographic position of uterus is approximately at the level of IV to VII lumbal vertebra, for *cornu uteri*, and for *cervix* along to I sacral vertebra, at the lining of abdominal to pelvic cavity, depending of reproductive stage and hormonal status [6-8]. Receptive doe exhibits a higher estrogen level and a higher number of large ovarian follicles [5], resulting in clinical signs of estrous: reddish and edematous vulva and *lordosis* [9]. As a reflexively ovulating species, rabbit preovulatory surge of luteinizing hormone (LH) is induced by sensory and neuroendocrine stimuli. LH concentrations began to raise 30 min *post coitum* [10]. Immediate release of LH from the anterior pituitary reaches the maximum after 1 h, which results in ovulation [11]. After the ovulation in rabbits has been induced [1,12,13], it starts 10-12 h later [14], and until the *corpora lutea* are formed, estrogen is dominant ovarian hormone [15]. Epithelial lining of cervical mucosa is a simple columnar epithelium with ciliated cells and goblet cells. Cervical mucosa forms the primary, secondary and tertiary folds, which do not change their appearance during the reproductive stages. Ciliated cells show small apical microvilli, between the cilia, while goblet cells are mucous secreting cells, with large membrane bound vesicles and dense cytoplasm [16]. Columnar epithelium in the upper FRT has strong network of tight junction, but high levels of estrogens lead to its relaxation and increase the permeability of the epithelium and possible degradation of its barrier function [10,17]. That morphological event creates higher susceptibility to infection and requires tissue and immune remodeling. Estrogen receptors in lymphocytes mediate estrogen induced proliferation of T lymphocytes and NK cells, but not B lymphocytes [18]. Endometrial growth is significant for this proliferative phase, but maximal endometrium development is evident in progestational phase. *Lamina propria mucosa* consists of a connective tissue network with fever glands and vascular and lymphoid vessels. The endocervical glands produce mucus, which make a physical barrier to pathogens and potential ascending infection [19]. The products of their secretory activity are dissimilar trough the stage of reproductive cycle: estrogenic mucus is thin and watery, with low viscosity, while progestational mucus is thick and viscous [17]. This different consistence of the mucus facilitates sperm movement to the upper FRT, or impedes the movement of material from low to upper part of this track, on the other hand. Morphological feature of uterine glands also differs through the stage [20-22]. At active, secretory state uterine glands assume highly coiled form. Lumen of the glands increase and presence of

glycoprotein secretory products indicates preparation for expected fertilization and implantation. Progesterone is an immunomodulatory molecule, which activity is connected with activation induced appearance of progesterone binding sites in the lymphocytes [23]. Unique immune cell phenotypes in FRT are different from those at other mucosal sites; even they are a part of mucosal immune system. Immune system throughout FRT is regulated by sex hormones, which directly or indirectly modulate recruitment and activation of lymphocytes [24]. The level of ovarian hormones significantly influences the distribution and the number of immune cells in FRT of rodents [25], rabbits [26] and humans [19,27,28].

The aim of this study was to investigate the histological changes and the changes in localization and number of immune cells in rabbit female reproductive tract following different reproductive stages.

MATERIAL and METHODS

Our study was carried out on female Californian rabbits aged 4-6 months and weighing 3500-4200 g. Animals were kept in an individual cage system, under the same environmental conditions, with free access to water and feeding *ad libitum*. Three groups with five animals in each were formed. Unmated female rabbits with clinical signs of estrous (red-colored vulva and *lordosis*) were selected as group I. Rabbits with clinical signs of estrous which were mated in order to induce ovulation formed group II and were sacrificed 12 h after mating. Group III consisted of gravid rabbits that were sacrificed on day 15 of gravidity, where day of mating was counted as day 0 and the next day as the 1st day of gravidity. Tissue samples of ovaries, *cornu uteri* and *cervix* (each side of organs) were collected, fixed in neutral 10% buffered formalin solution, embedded in paraffin, following the standard procedure, sectioned at 5 μm thick (10 transverse sections/organ) and stained with standard H&E technique. The phase of reproductive cycle was confirmed according to the ovarian morphology, as estral phase, post-ovulation phase and gravidity *(Fig. 1)*.

Immunohistochemistry was performed on serial cryosections of the lymph node, which were used as the positive control, while stained *cervix* and uterine horns tissue samples were used for detecting immune cells. Tissue samples, frozen in liquid nitrogen, were cut at cryotome (Leica), in transverse cryosections of 5 μm, 10 section/organ, at -24°C. After that, the samples were air dried for 12-16 h, at 21°C, fixed in cold acetone for 10 min and stored at -20°C until further use. For detection of T- and B-cells immunoperoxidase technique was used. T-cells were detected using mouse anti-rabbit T-lymphocytes monoclonal antibody *(Mouse anti Rabbit T Lymphocytes, clon KEN-5+, Catalog Number: MCA800G, AbD Serotec, A Bio-rad Company, Raleigh, North Carolina, USA)*, which recognizes rabbit T cells and binds to CD5+, whereas B-cells
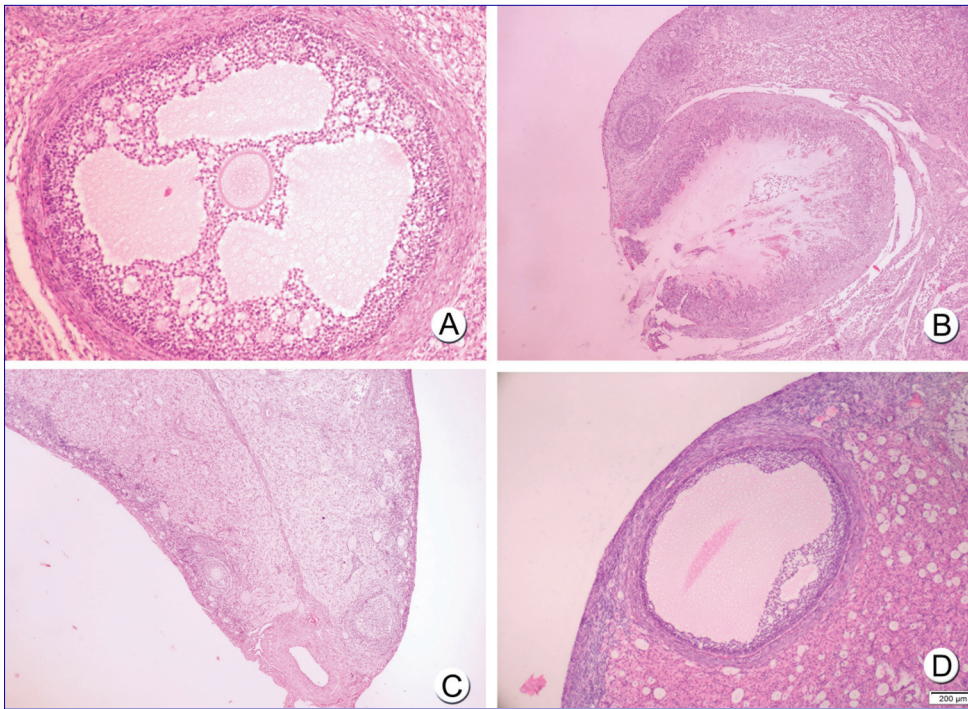
Fig 1. *Ovarium* at different reproductive stages

A: estrous, H & E, x400, B: ovulation, H & E, x40, C: pregnancy, H & E, x40, D: ovulatory follicle with diameter approximately up to 1100 µm, H & E, x40

Şekil 1. Farklı üreme dönemlerinde ovaryum

A: östrus, H & E, x400, B: ovulasyon, H & E, x40, C: gebelik, H & E, x40, D: çapı yaklaşık 1100 µm olan ovulasyon folikülü, H & E, x40

were detected with mouse anti-rabbit IgM monoclonal antibody *(Mouse anti Rabbit IgM-B cell Marker, clon NRBM, Catalog Number: MCA812GA, AbD Serotec, A Bio-rad Company, Raleigh, North Carolina, USA)*. Polyclonal goat anti-mouse IgG antibody conjugated with peroxidase was used as secondary antibody *(R&D Systems)*. For visualization the antigen-antibody complex, the slides were incubated with the peroxide substrate, *(DAB Peroxidase Substrate Tablet set, Sigma-Aldrich)*. Slides were lightly counterstained with Mayer's haematoxylin and cover slipped using Kaiser's glycerol gelatin as mounting media. Cryosections of *uterus* and *cervix*, stained without primary antibody incubation were used as negative control.

Histological and morphometric analyses were done by light microscopy (Olympus BX53), with compatible digital camera (Olympus UC50). Morphometric measurement of epithelial height was taken with computer-assisted image analysis system Olympus cellSens microscope imaging software, with magnification x40. At every section 15 measurements were taken, resulting in 150 measurements/animal. Reference area for T- and B-cell counting was defined using an ocular micrometric scale and microscopic ratio (RA=17.200 µm²) with the magnification x40 at 50 areas (5 defined area/section). The results were represented as the number of positive cells/area. The number of immunopositive cells was presented as mean ± standard deviation (SD). Means of the number of T-cells in *uterus,* during the different stages of reproductive cycle, were analyzed statistically by Kruskal-Wallis method, followed by *post-hoc* Dunn's test, using Statistica v.6.0 (Statsoft USA). The differences between mean values were considered statistically significant if the P value was below 0.05. The investigation was done with the Permission of the Ethics Committee of Faculty of Veterinary Medicine, University of Belgrade, No 01-14/7.

RESULTS

Lamina epithelialis mucosae uteri varied in its thickness and height from stage to stage of reproductive cycle, depending also on anatomical part of *uterus*. A tall columnar ciliated and non-ciliated epithelial cells differed in their morphological appearance. Non-ciliated cells had dense cytoplasm in their basal portion, while secretory material occupied their apical processes formed as protrusion. The morphological profile of ciliated cells differed to previous one with slightly staining cytoplasm and larger size. The morphological change of *gll. uterinae* was also evident considering the shape, which lenghten within endometrial stroma and changed their initial straight tubular configuration to more sinous one.

The height of epithelial cells were increased *(Table 1)*, and endometrium became greatly thickened in post-ovulatory phase. Ciliated cells were the more prominent type of cells. Curled endometrial glands were spread through the loosely arranged connective tissue to the

Table 1. *Epithelial height in uterus, at different reproductive stages*
Tablo 1. *Farklı üreme dönemlerinde uterus epitel uzunluğu*

Uterine Segment	Estrous	Ovulation	Pregnancy
Cornu uteri (µm)	14.086±3.337[a]	16.339±2.219[b]	15.512±2.090[c]
Cervix (µm)	32.709±5.446[a]	37.520±5.166[b]	20.137±2.227[c]

Different superscript letters [abc] *in the same row represent significantly different value (P<0.05)*
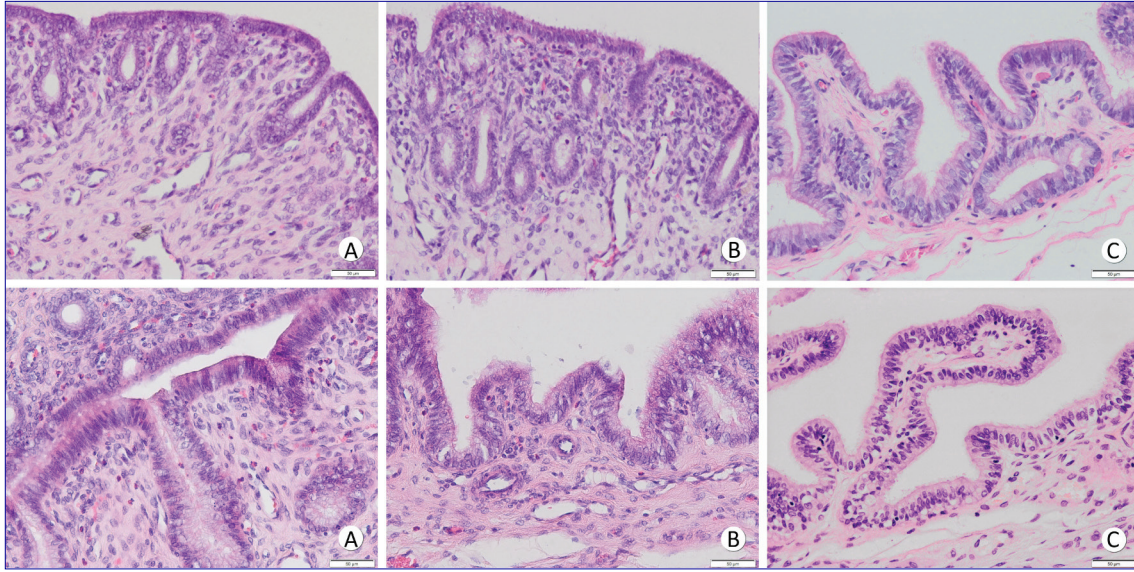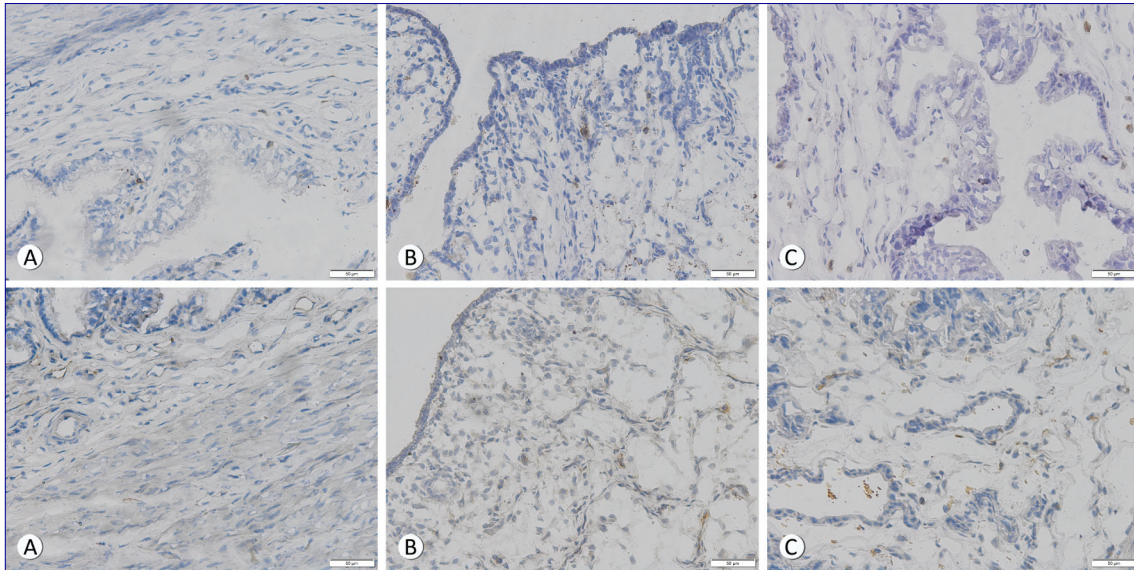
Fig 2. *Uterus* at different reproductive stages
Upper row: *cornu uteri,* **Lower row:** *cervix uteri,* A: estrous, B: ovulation, C: pregnancy, Scale bar 50 μm, H & E

Şekil 2. Farklı üreme dönemlerinde uterus
Üst sıra: cornu uteri, **Alt sıra:** serviks uteri, A: östrus, B: ovulasyon, C: gebelik, Ölçek 50 μm, H & E

Fig 3. Distribution of T- and B-cells at *cornu uteri*
Upper row: T-cells, **Lower row:** B-cells at *cornu uteri,* A: estrous, B: after ovulation, C: pregnancy. Higher density of immunopositive cells was recorded for T-cells in estrous and post ovulatory stage. Scale bar 50 μm, chromogen diaminobenzidine, counterstain hematoxylin

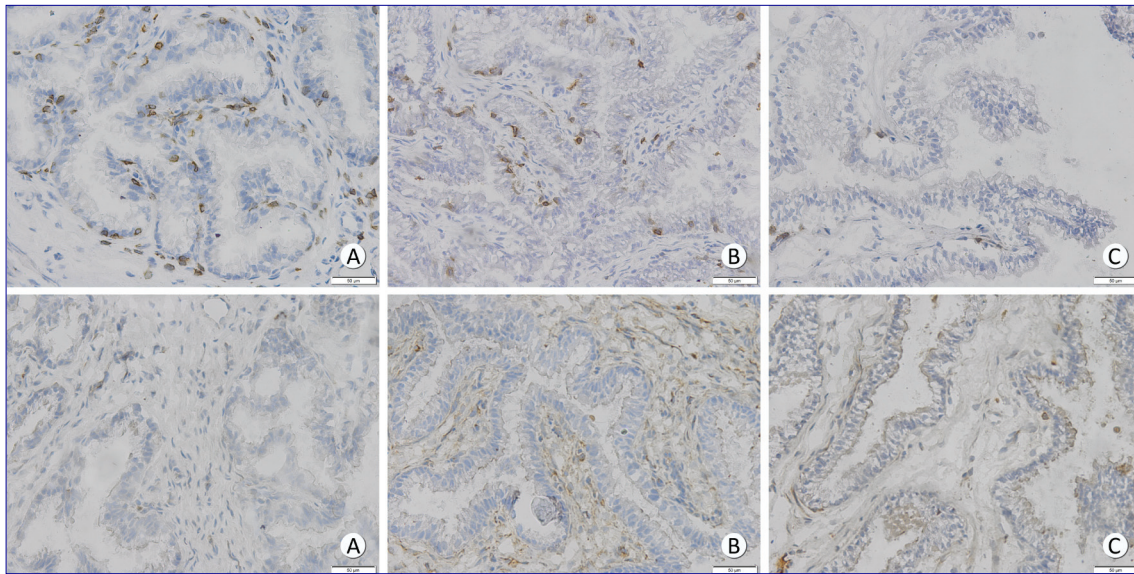**Şekil 3.** T ve B hücrelerinin cornu uterideki dağılımı
Üst sıra: T hücreler, **Alt sıra:** cornu uteride B hücreler, A: östrus, B: ovulasyon sonrası, C: gebelik. Östrus ve ovulasyon sonrası dönemlerde yüksek yoğunlukta T hücreleri gösterir immunpozitivite. Ölçek 50 μm, kromojen diaminobenzidine, zemin boyaması hematoksilen

myometrium, enlarged in their size and with dilated lumen. At this stage, the presence of lymphocytes in stroma was noticed. During pregnancy, epithelium became highly columnar and glandullar cells increased in height *(Fig. 2)*.

Cervical epithelium, consiting of cilliated cells, larger in size and with centrally or apically located nucleus, and secretory cells, narrow and with basally located nucleus, showed activity changes as a consequence of hormonal status. During ovulation, secretory cells increased their activity and secretory granules were noticed on their surfaces. During pregnancy, cervical mucosa formed the primary, secondary and tertiary folds; its columnar epithelium was edematous and the capillary network was particularly expressed *(Fig. 2)*.

Fig 4. Distribution of T- and B-cells at *cervix uteri*
Upper row: T-cells, **Lower row:** B-cells at *cervix uteri,* A: estrous, B: after ovulation, C: pregnancy. The highest number of labeled immunopositive cells was noticed for T-cells in estrous. Scale bar 50 µm, chromogen diaminobenzidine, counterstain hematoxylin

Şekil 4. T ve B hücrelerinin serviks uterideki dağılımı
Üst sıra: T hücreler, **Alt sıra:** serviks uteride B hücreler, A: östrus, B: ovulasyon sonrası, C: gebelik. En fazla immunpozitif hücreler östrusta T hücrlerdi. Ölçek 50 µm, kromojen diaminobenzidine, zemin boyaması hematoksilen

Table 2. Number of T and B lymphocytes per RA in cervix and cornu uteri, at different reproductive stages

Tablo 2. Farklı reproduktif dönemlerinde serviks ve kornu uterideki her bir RA'da T ve B lenfosit sayıları

Uterine Segment		Estrous	Ovulation	Pregnancy
Cornu uteri	T	1.280±0.131[a,1]	1.440±0.107[a]	0.200±0.057[b,1]
	B	0.280±0.075	0.800±0.128	0
Cervix	T	2.120±0.123[a,2]	1.280±0.107[b]	1.260±0.106[b,c,2]
	B	0.040±0.028	0.520±0.082	0.320±0.078

Different superscript letters [abc] in the same row represent significantly different value (P<0.05), different superscript numbers [1,2] in the same column represent significantly different value (P<0.05)

Following estrous, T-cells, round in shape, were located in subepithelium of *cornu uteri*, while B-cells were present around the blood and lymphatic vessels in the stroma. After ovulation, only a few T-cells were found separately in epithelium, while the majority was found at *lamina propria mucosae* and around blood vessels, along with scant B-cells around the blood and lymphatic vessels. During the pregnancy, the number of T-cells decreased and the presence of B-cells was only barely detected *(Fig. 3)*.

The number of T-cells in *cornu uteri* at estrous and in post-ovulatory stage was significantly higher (P<0.05) compared to their number in pregnancy *(Table 2)*.

During estrous, T-cells were present separately or in cell clusters under the epithelium, in the stroma of mucosal folds in *cervix* and around lymphatic vessels. This T-cell distribution was similar to the one in the post-ovulation phase. During pregnancy, both T- and B-cells were located under the cervical epithelium in very small numbers *(Fig. 4)*.

Although the localization of T-cells was similar in estrous and after ovulation, their number differed significantly (P<0.05). The number of T-cells was higher in estrous compared to pregnancy (P<0.05). Number of T-cells in *cervix* at estrous showed significant difference compared to the number in *cornu uteri* (P<0.05), and also in pregnancy. After ovulation, there was no significant difference in cell numbers between these regions *(Table 2)*.

DISCUSSION

The maintenance of the immune system in FRT is important not only in prevention of potential diseases, but also in limiting exposure to seminal male antigens, deposited during mating. Transforming growth factor β (TGFβ), contained in seminal plasma, stimulates post-coital immune response to pathogens and induces immune tolerance to those male antigens [29,30]. Although the *uterus* is not an immunologically privileged site, the immune response of genital tract could be modified toward immune tolerance [31,32]. Partutition is, on the contrary, characterized by influx of immune cells, that promote the re-establishment of the immune process [33].

Normal healthy FRT possesses various immuno-competent cells, such as macrophages, neutrophils, natural killer cells, Langerhans cells, lymphocytes, with T-cells and scarce B-cells [24,33,34]. A significant population of leukocytes in endometrium consists of T-cells (about 50%), where 2/3 of these cells are CD8^{+} T cells [35,36]. CD4^{+} and CD8^{+}

T-cells are located in stroma of lower genital tract as single cells or cell clusters, whereas in *uterus* these cells are organized as lymphoid aggregates. In human and mouse reproductive tracts, T-cells also represent the most numerous lymphocyte cell population [25,35]. In this study, we analyzed the presence of T- and B-cells in rabbit FRT. We found that CD5+ T-cells in rabbit endometrium, as a single cells or clusters, were located beneath epithelium. Higher number of these cells was observed at endometrial stroma. In cervical folds and its stroma, CD5+ T-cells were also detected. These findings are in correlation with previous publishing data [23,26]. Regarding the distribution of B-cells, we found that these cells were sparsed through endometrium stroma and around blood vessels in *cervix* and uterine horns, which is in correlation with findings in mice [37] and humans [36].

It is well known that the immune system in genital tract is regulated by sex hormones, produced by ovary during the reproductive cycle, so as to encompass the reproductive process [24]. The number of immunocompetent cells depends on the hormonal status and increases during proliferative phase. The number of T-cells in humans increases from the beginning of the menstrual cycle and reaches its maximum at the end of the proliferative stage [31,36]. Estrogen, as a regulator of this mechanism [38], provides *uterus* with better resistance to infection at estrous, but not in diestrous [39], by regulating the number, function and distribution of immune cells and antigen presentation in genital tract. The presence of major histocompatibility complex (MHC II+) molecules was expressed more prominently in uterine body of mares than in the *cornu uteri* during the estrous [39]. In this study, we found a significantly higher number of T-cells in *cervix*, at estrous, as compared to other phases of the reproductive cycle. This finding differs from other findings in rabbits [26]. The increase in T-cells after ovulation could be a result of a differently defined control group. That group conisted of unmated, healthy female rabbits, with no clinical signs at estrous [26], which differs from the control group in our study.

Progesterone, also known as a natural immunosupresor, is essential for adequate maternal immune response to the fetus. Progesterone-mediated immunosupresive effect is achived by the downregulation of CD80, CD86 and MCH II+ molecules on B-cells, and by supressing its antigen presentation. According to findings in humans and rodents, lower numbers of immune cells such as CD4+ T-cells and dendritic cells are detected in *uterus* than in *vagina* during the dioestrous, and this could be a consequence of progesterone activity [25,33,37,40]. The presence of a low number of IgM plasma cells at rabbit *cervix* was recorded under the epithelium, or in stroma, while those cells were located mostly around blood vessels and in endometrial stroma [26]. It is known that the number and the localisation of IgM positive cells are not affected by the reproductive stage. Our study, along with a previous one [26], supports

these findings. However, the lowest number of B-cells in gravid uterine horns, coupled with the lowest number of T-cells, described in our study, indicated a possible existence of immune tolerancy. Considering the findings that CD5 is overexpressed on regulatory T-cells (Treg) and regulatory B-cells [41], and that αβTCR+CD4-CD8- T- cells with regulatory function are dominant in mouse genital tissue [32], the presence of T-cells in *cervix* suggests that they may represent Treg cells. As αβTCR+CD4-CD8- T-cells are regulatory cells, and only a small proportion of rabbits B cells expresses CD5 [42], cells positive for CD5 staining in this investigation could be Treg cells. Those cells modulate immune reactivity in homeostatic and inflamatory conditions and lead to an enhanced immune response. The lower number of T-cells in post-ovulatory stage suggests that immune response in uterus gives way to immune tolerance, expecting a possible gestation. The assumption mentioned above remains to be confirmed in our further studies.

The presence of immunocompetent cells also depends on the anatomical compartments of reproductive tract [40]. Cervical and vaginal epithelial cells, together with macrophages and neutrophiles, present the first line of defense against pathogens [25]. *Cervix*, as the anatomical part of reproductive tract, has a more intense immunological activity in humans [19], due to the highest number of macrophages, CD4+, and especially CD8+ T-cells [35]. At cervical transformating zone, the accumulation of CD8+ and TIA+ cells indicated that this anatomical site is a strong immune barrier for ascending pathogens. Our finding of a significant number of T-cells in rabbit *cervix* under the epithelium and along the stroma suggests a possible migration of these cells into epithelium and implicates significant cervical reactivity, compared with uterine horns.

Lamina epithelialis mucosae and *lamina propria mucosae uteri* were exquisitely responsive to steroid hormonal stimulation or deprivation, which resulted in different morphological profiles through the reproductive phases. The number of T- and B-cells also showed considerable variation according to the reproductive stage. The findings that this immunological activity was significantly different in estrous compared to other reproductive stages indicates the principal role estrogen has in the regulation of adaptive immune response in rabbits. Similar findings were described in humans [24,34,36].

CONFLICT OF INTEREST STATEMENT

The authors declare that that there is no conflict of interest.

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