Short Paper

Co-relation of estrous cycle phases with uterine bacterial and fungal flora in non-pregnant female laboratory rabbits

Mogheiseh, A.[1*]; Derakhshandeh, A.[2]; Batebi, E.[3]; Golestani, N.[4] and Moshiri, A.[3]

[1]Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [3]Veterinary Medicine Private Practitioner, Shiraz, Iran; [4]Graduated from School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Correspondence: A. Mogheiseh, Department of Clinical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran. E-mail: mogheiseh@yahoo.com

Summary

This study was designed to investigate the relationship between the estrous cycle phases with uterine bacterial and fungal flora in non-pregnant female rabbits. Thirty laboratory mature multiparous rabbits were used for this purpose. Samples from uterine lavage for culture of bacteria and fungi were collected at different stages of estrous cycle (based on vaginal cytology), and histopathological observations were evaluated based on the scoring system used for defining the infection of the uterus. Various types of bacteria and fungi were isolated from rabbits at all stages of estrous cycle. The widest variety of bacteria and fungi was isolated at Di-estrous stage and the lowest variety was detected at estrous stage. *Klebsiella oxytoca* as well as yeast have been isolated at all stages of estrous cycle. This study showed that infection with *K. oxytoca* and yeast had no relationship with different stages of estrous cycle but other bacteria and fungus were associated with one or more stages of the estrous cycle in rabbits.

Key words: Bacterial flora, Estrous cycle, Female rabbit, Fungi, Uterus flushing

Introduction

Rabbits are not only one of the most commonly used lab animals for experimental studies but they are also used to produce meat and leather. Cases of contagious infections have been reported in reproduction and breeding centers of rabbits which lead to widespread economic damages (Jacques *et al*., 1986). These infections induce general female reproductive diseases which lead to abortion, subfertility and other reproductive disorders (Jacques *et al*., 1986). *Staphylococcus aureus* has been reported to be the most serious problem in these farms and this organism has been isolated from 70% of the infected animals (Sequra *et al*., 2007). *Pasteurella* species have been found the most prevalent bacteria in cases of pyometra and pneumonia (Sequra *et al*., 2007). Both *Pasteurella multocida* and *S. aureus* have been shown to be general factors for the testicular inflammation and endometritis in rabbits (Sequra *et al*., 2007). It has been reported that these infections decreased fertility (Sequra *et al*., 2007). Another study showed that staphylococci based mastitis was one of the main factors of culling of adult rabbits in farms (Viana *et al*., 2011).

A number of reports in the literature have focused on the genital micro flora of different animal species such as mouse (Tregier and Homburger, 1961), rats (Larsen *et al*., 1976), rabbits (Jacques *et al*., 1986), baboons (Shangalis *et al*., 1979), dogs (Baba *et al*., 1983), horses (Scott *et al*., 1971) and cows (Messier *et al*., 1984). Jacques *et al*. (1986) isolated and determined the bacterial flora of reproductive tract including vagina, cervix and uterus of rabbits, using tissue sampling technique without considering different stages of estrous cycle. Thus, this study was designed to investigate the relationship between phases of estrous cycle with bacterial and fungal flora of the uterus in non-pregnant female white New Zealand laboratory rabbits.

Materials and Methods

Animals

Thirty white New Zealand multiparous laboratory rabbits were used. The average female age was above 12 months old, and all of them were sexually and skeletally mature. Their maturity was confirmed by radiography, physical and ultra-sonographical examination of their sexual organs. All rabbits had birth certificate. Their average weight was between 2.5 to 3.2 kg (mean 2.84 ± 0.32 kg). Prior to their use, all rabbits were housed individually from three to four weeks in stainless steel cages. The animal were fed commercial rabbit diet (hey pellet) and water *ad libitum* and had free access to food and water. Their room temperature ranged from 20 to 25°C and relative humidity from 50% to 69% with controlled light (12:12 h light:dark cycle).

Sample collection and preparation of vaginal smears

The vaginal smears were collected using 15 cm long, cotton swab. The swab was entered through the vagina to collect the discharges from the anterior and mid part of the vagina. The swab was rolled in vagina with the circular pattern of 360 degree, removed and rolled over a slide to make a thin smear of the vaginal discharge. The smear was air dried and was then fixed using methanol for 10 min. The fixed smears were then stained by 1:10 diluted Giemsa stain in distilled water for 20 min (Ypsilantis et al., 1996).

Classification of the vaginal smears

The method has previously been described by Ypsilantis et al. (1996). Briefly, the vaginal smear was examined at magnification of ×400. The vaginal epithelial cells were classified into four types, according to the classification system (Parabasal, Intermediate, Superficial, Anuclear) described in other mammals (Ypsilantis et al., 1996). The authors have described this classification in Fig. 1. Briefly, pro-estrous (A and B): the smear is clear and dominated by intermediary cells, superficial cells and keratinized cells. The estrous (C and D): at this stage, the smear consists almost entirely of keratinized cells that lie singly or form groups. Superficial cells can also be observed. At Met-estrous (E and F) the cellular population is dominated by leucocytes, often in large numbers and intermediary cells. At Di-estrous (G and H), there are lots of little materials together with few intact cells and some basal and intermediary cells.

Euthanasia and ethics

The investigators who undertook the clinical observations, measurements and analysis of the results in the present study were unaware of the experimental design and grouping details. The approved methods were used in anesthesia and euthanasia of the animals. The animals were euthanatized by injection of a combination of 1 mg/kg gallamine triethiodide (Specia Co., Paris, France), 15 mg/kg ketamine (Ketamin 10%, Alfasan Co., Woerden, Netherlands), 2 mg/kg xylasin (Xylazin 2%, Alfasan Co., Woerden, Netherlands), and 1 mg acepromazine maleate (Acepromazin 1%, Neurotranq, Alfasan Co., Woerden, Netherlands) in the heart of each rabbit. The study was approved by the local ethical committee of our faculty, according to the standards of "Principles of Laboratory Animal Care" (Oryan and Moshiri, 2012).

Flushing, tissue sampling and histologic preparation

After euthanasia of the rabbits, the uterus was dissected and removed. Because of specific antomy of rabbit's uterus (bicornuate duplex uterus), each horn was flushed with 3 ml of normal saline separately. Then samples of two horns were pooled. The pooled sample of each uterus was transferred into a sterile tube and was maintained at -20°C. The mid part of the uterine horns was then necropsied and transferred into neutral buffered 10% formalin. After fixation in 10% neutral buffered formalin, the samples were washed, dehydrated, cleared, embedded in paraffin wax, longitudinally sectioned at 4-5 μm thickness, stained with haematoxylin and eosin and examined by a light microscope (Olympus, Tokyo, Japan) (Oryan et al., 2012). Various stages of estrous cycle of the rabbits were determined, using histology sections together with the vaginal smears. In histology the following criteria were used to define the stage of estrous cycle: pro-estrous: the endometrial epithelium should be composed of medium-sized, tall columnar
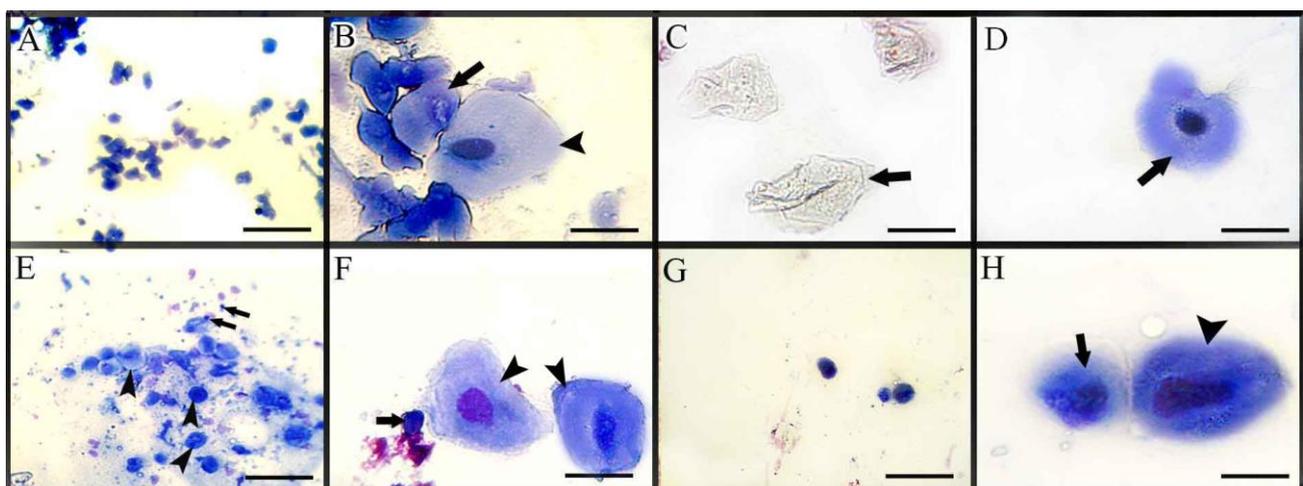

Fig. 1: Vaginal cytology. **A** and **B**: Pro-estrous cycle. The smear is clear and dominated by cells. These consist of intermediary cells (arrow), superficial cells (arrow head) and keratinized cells. **C** and **D**: At this stage of the estrous cycle, the smear consists almost entirely of keratinized cells that lie singly or form groups (arrow in Fig. 2C). Superficial cells can also be observed (arrow in Fig. 2D). **E** and **F**: At this stage (Met-estrous) the cellular population is dominated by leucocytes (arrows), often in large numbers and intermediary cells (arrows head). **G** and **H**: At this stage of oestrous cycle (Di-estrous), there are lots of little materials that can be seen. Few intact cells can be found. Some basal (arrow) and intermediary cells (arrow head) can be seen, (scale bar for A, E, G= 25 μm and for B, C, D, F, H= 10 μm, color staining: Giemsa)
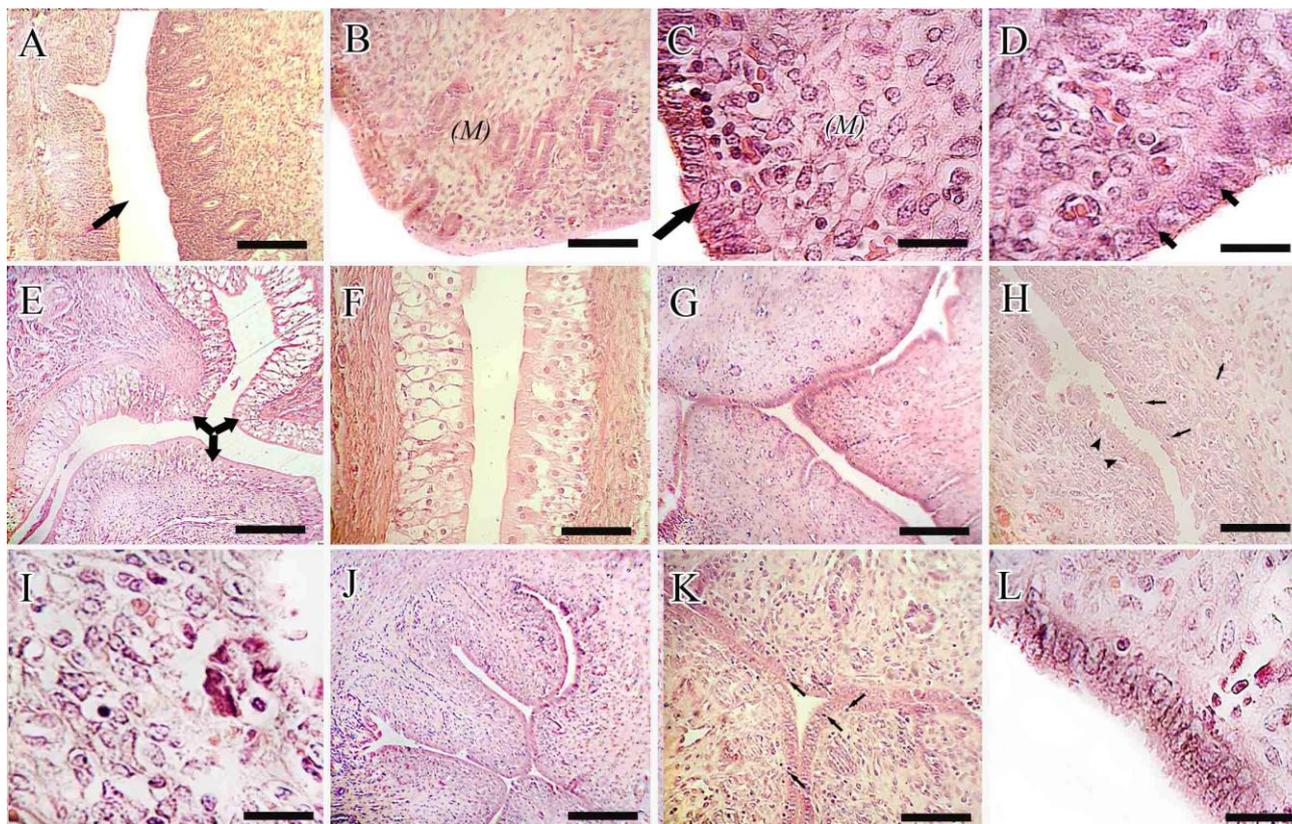
Fig. 2: Light microscopy of the endometrial tissue with description of the oestrous cycle. **A-D**: Pro-estrous: the endometrial epithelium is composed of medium-sized, tall columnar cells (C and D). Low number of mitotic figures (*M*) is seen in the luminal uterine epithelium. Generally, the lumen showed a marked dilatation toward the end of the stage (A). (A) Small luminal dilation is seen (arrow), (C and D) show cuboidal to columnar epithelial cells (arrows). **E-F**: Estrous: appearance of cellular degeneration/necrosis in the glands. Luminal dilation may persist into late estrous (arrows). **G-I**: Met-estrous: the endometrial epithelium is reduced in height compared with oestrous. Epithelial cell degeneration/apoptosis observed during oestrous persist during this stage. Epithelial regeneration, characterized by increased mitotic activity (*M*), begins during Met-oestrous. Fig. 1H shows the epithelial mitotic figure (arrows) and epithelial necrosis (arrow head). **J-L**: Di-estrous: small slit like lumen. The luminal and glandular epithelium are reduced in height; both are composed of small, low columnar cells. Occasional apoptotic/degenerate epithelial cells may be present and mitotic figures (*M*) are numerous. Increased mitotic figure is seen in Fig. 1K (arrows), (scale bar for A, B, E, G, H, J and K= 100 μm and for C, D, F, I and L= 25 μm, color staining: H&E)

cells. Low number of mitotic figures should be seen in the luminal uterine epithelium and generally, the lumen shows a marked dilatation toward the end of this stage (Westwood, 2008). Estrous: appearance of cellular degeneration/necrosis in the glands should be distinguishable. Luminal dilation may persist into late estrous (Westwood, 2008). Met-estrous: the endometrial epithelium should be reduced in height compared with estrous. Epithelial cell degeneration/apoptosis observed during estrous persist during this stage. Epithelial regeneration, characterized by increased mitotic activity, begins during Met-oestrous. Di-estrous: small slit like lumen (Russel Westwood, 2008). The luminal and glandular epithelium are reduced in height; both are composed of small, low columnar cells. Occasionally apoptotic/degenerated epithelial cells may be present and mitotic figures are numerous (Figs. 2A-L).

Microbiological and mycological examination

Each sample obtained from the uterine flushing was centrifuged at a rate of 3000 ×g for 10 min. The sediments were used as inocula. Each sediment was inoculated onto 5% sheep blood and MacConkey agar culture and the plates containing the samples and culture media were incubated at 37°C for 24-48 h. After growth of the discrete colonies, they were observed under light microscopy and the results were recorded. Gram staining and other routine biochemical tests were further employed with the aim of differentiating the bacteria based on standard procedures has previously been described (Devriese, 1984).

The same samples were inoculated onto Sabouraud's dextrose agar (SDA), incubated at 25°C for 2-3 weeks. Chloramphenicol was used in the agar media for initial fungal isolation. The cultures were examined daily for any mycobiotic growth during the incubation period. Visual examinations of the fungal colonies were made, and their colonial morphology or characteristics, such as texture, pigment and rate of growth on media, were recorded. Morphology of the fungi was identified by examining a small aliquot of the growth in lactophenol blue under dry objective microscope. Fungal isolates were identified to the genus level. Duplicate culture was used for each sample.

Table 1: The result of bacterial and fungal cultures of uterine flushing of rabbits in different stages of estrous cycle

Stage of estrous cycle	Bacteriology	Mycology	Histopathology
Estrous (n=3)	*Klebsiella oxytoca*	Yeast *Geothericum*	2
Met-estrous (n=6)	*Klebsiella oxytoca* *Micrococcus* *Bacillus*	*Cladosporium* *Scopolariopsis* Yeast *Alternaria*	14
Di-estrous (n=14)	*Staphylococcus aureus* *Staphylococcus hyicus* *Staphylococcus intermedius* *Staphylococcus lentus* *Klebsiella oxytoca* *Bacillus*	*Cladosporium* Yeast *Aspergillus* *Scopolariopsis Bipolaris*	8
Pro-estrous (n=7)	*Staphylococcus aureus* *Staphylococcus-intermedius* *Klebsiella oxytoca*	*Geothericum* *Scopolariopsis* Yeast	19

Statistical analysis

Statistical analysis (independent sample t-test) was performed to analyze the histopathologic data and significant difference was determined as P<0.05.

Results

Before euthanasia, none of the animals were clinically ill and their health was confirmed by the small animal practitioner of the laboratory animal house. The animals had normal weight gain and their appetite was normal.

According to the histologic samples, maximum numbers of leukocytes were recorded at pro-estrous and the minimum numbers were observed at estrous (P=0.001) (Table 1).

The result of the aerobic and anaerobic and mycotic cultures from uterine flushing of the rabbits is given in the Table 1. Widest variety of bacteria and mycotic organism was seen at Di-estrous stage of oestrous cycle and the lowest varieties were seen on estrous stage of oestrous cycle. *Klebsiella oxytoca* as well as yeast was isolated at all stages of estrous cycle but *Micrococcus* has been isolated only at Met-estrous stage. Species diversity of *Staphylococcus* was higher at Di-estrous than other stages (Table 1).

As shown in Table 2, the most bacteria isolated from the rabbits' uterine lavage was *K. oxytoca*, *Micrococcus* and *Bacillus* were the lowest bacteria isolated from the animals, so that 60% of the rabbits showed *K. oxytoca* and 6.6% and 10% of the animals showed *Micrococcus* and *Bacillus* in their samples, respectively. 22.6% of the animals showed staphylococci and *S. aurous* (10% of the animals showed this organism) was the most important types with higher incidence among other types of this bacteria. The most mycotic organism isolated from the rabbits' uterine lavage was Yeast (40%) and *Cladosporium* (23%) and the lowest was *Bipolaris* (3%) (Table 3). *Alternaria*, *Aspergillus*, *Geothericum* and *Scopolaris* showed the same incidence (between 6.6 to

10% of the animals showed these organisms in their samples) and few animals showed them in their samples.

Table 2: Number of positive rabbits in bacterial culture based on type of bacteria

Bacteria	Number of positive rabbits in culture (%)
Bacillus	3 (10%)
Klebsiella oxytoca	18 (60%)
Micrococcus	2 (6.6%)
Staphylococcus aureus	3 (10%)
Staphylococcus hyicus	1 (3%)
Staphylococcus intermedius	2 (6.6%)
Staphylococcus lentus	1 (3%)

Table 3: Number of positive rabbits in fungal culture based on type of fungus

Fungus	Number of positive rabbits in culture (%)
Alternaria	3 (10%)
Aspergillus	2 (6.6%)
Bipolaris	1 (3%)
Cladosporium	7 (23%)
Geothericum	2 (6.6%)
Scopolariopsis	3 (10%)
Yeast	12 (40%)

Discussion

In this study, different types of bacteria and fungi were isolated and identified at various stages of estrous cycle of all 30 female non-pregnant laboratory rabbits. At the Di-estrous stage, a more and wider variety of bacteria and fungi were found, however, on the other hand, the lowest variety of bacterial and fungal growth was seen in the estrous stage.

The lower amount of microbial flora at estrous and higher amount of microbial flora at Di-estrous observed in the results of the present study can be due to variation of the number of leucocytes at different stages of estrous cycle. It has been shown that, the highest number of leucocytes in estrous stage and the lowest number of leucocytes at diestus stage is present during the course of the cycle. The results of the present study are in

accordance with those of the Schultheiss *et al*. (1999) who showed that bacteria were isolated from 40% of Di-estrous samples of the female canine reproductive tract but they were in lower number at estrous stage. The results of the present study are not in agreement with Olson *et al*. (1978) who showed similar results at different stages of the estrous cycle, when they isolated bacteria from the uterine samples of adult dogs. Baba *et al*. (1983) found that the total bacteria of the vaginal samples in estrous stages were higher than those of the anesterous, pregnancy and postpartum stages in adult dogs. Watts *et al*. (1996) reported that bacteria were always found in the uterus during pro-estrous and estrous (12 positive in 12 cultures) and they were rarely seen at other stages of the reproductive cycles in the bitch.

At histological evaluation, number of inflammatory cells was counted and no unusual histological changes were seen in all samples. Inflammatory cells existed in different stages of reproductive cycle, especially at estrous and met-estrous stages. This condition is not pathologic and is normal physiologic condition except for the situation with the higher number of neutrophils (more than 25% of the total cellularity is neutrophil). In a normal uterus, infiltration of the inflammatory cells, particularly neutrophils, should not be excessive in Di-estrous stage. The investigators used continuous changes in the histological appearance of various components of the reproductive tract during the estrous cycle to determine a particular phase of the estrous cycle in rats (Westwood, 2008). They have described little inflammatory cell infiltration in pro-estrous and higher infiltration of the inflammatory cells in estrous stages (Westwood, 2008).

Unlike other animal species which are known to support an important genital tract mucosal micro flora, few bacteria were isolated from the rabbits' genital tract mucosal surfaces. The predominant constituents of rabbit uterus microflora were *K. oxytoca* (18/30, 60%), *Staphylococcus* (6/30, 20%), *Bacillus* (3/30, 10%) but, in the study of Jacques *et al*. (1986), they have isolated microorganisms (mainly coagulase-negative staphylococci) from 45% of the uterine samples. There is continuing controversy over whether or not the normal human uterus is sterile (Skangalis *et al*., 1982), but in different animal species such as cow (Messier *et al*., 1984), horse (Scott *et al*., 1971), dog (Baba *et al*., 1983), mouse (Tregier and Homburger, 1961) and rat (Larsen *et al*., 1976), different bacterial species have been isolated from normal uteri. Moreover, this study has shown that bacteria have been isolated from the normal uteri of the laboratory animals and these results can be the start of future studies aiming to prove that normal uterus, even in optimum level of the healthy condition would not be sterile.

The predominant mycotic flora isolated from the rabbits' uterus, was Yeast (12/30, 40%). In other studies it has been reported that Yeast was found to be the predominant micro flora in human, and animals such as cow (Garoussi *et al*., 2007), dog (Cleff *et al*., 2005), horse (Chengappa *et al*., 1984) and monkey (Steel *et al*., 1999).

Hence, our results indicated that rabbits seem to process a relatively simple uterus micro flora and mycotic flora, regarding number and types of microorganisms, and therefore might be advantageously used for intra-uterine contraceptive devices microbial colonization studies and examination and quantitative culture results.

References

Baba, E; Hata, H; Fukata, T and Arakawa, A (1983). Vaginal and uterine microflora of adult dogs. Am. J. Vet. Res., 44: 606-609.

Chengappa, MM; Maddux, RI and Grer, SC (1984). Isolation and identification of yeasts and yeastlike organisms from clinical veterinary sources. J. Clin. Microbiol., 19: 427-428.

Cleff, MB; Lima, AP; Faria, RO; Meinerz, ARM; Antunes, TA and Araujo, FB (2005). Isolation of Candida spp from vaginal microbiota of healthy canine females during estrous cycle. Br. J. Microbiol., 36: 201-204.

Devriese, LA (1984). A simplified system for biotyping *Staphylococcus aureus* strains isolated from different animal species. J. Appl. Bacteriol., 56: 215-220.

Garoussi, MT; Khosravi, AR and Havareshti, P (2007). Mycoflora of cervicovaginal fluids in dairy cows with or without reproductive disorders. Mycopathologia. 164: 97-100.

Jacques, M; Olson, ME; Crichlow, AM; Osborne, AD and Costerton, JW (1986). The normal microflora of the female rabbit's genital tract. Can. J. Vet. Res., 50: 272-274.

Larsen, B; Markovetz, AJ and Galask, RP (1976). The bacterial flora of the female rat genital tract. Proc. Soc. Exp. Biol. Med., 151: 571-574.

Messier, S; Higgins, R; Couture, Y and Morin, M (1984). Comparison of swabbing and biopsy for studying the flora of the bovine uterus. Can. Vet. J., 25: 283-288.

Olson, PN and Mather, EC (1978). Canine vaginal and uterine bacterial flora. J. Am. Vet. Med. Assoc., 172: 708-711.

Oryan, A and Moshiri, A (2012). Recombinant fibroblast growth protein enhances healing ability of experimentally induced tendon injury *in vivo*. J. Tissue Eng. Regen. Med., 8: 421-423.

Oryan, A; Moshiri, A; Meimandi Parizi, AH and Raayat Jahromi, A (2012). Repeated administration of exogenous sodium-hyaluronate improved tendon healing in an *in vivo* transection model. J. Tissue Viability. 21: 88-102.

Schultheiss, PC; Jones, RL; Kesel, ML and Olson, PN (1999). Normal bacterial flora in canine and feline uteri. J. Vet. Diagn. Invest., 11: 560-562.

Scott, P; Daley, P; Baird, GG; Sturgess, S and Frost, AJ (1971). The aerobic bacterial flora of the reproductive tract of the mare. Vet. Rec., 88: 58-61.

Skangalis, M; Mahoney, CJ and O'leary, WM (1982). Microbial presence in the uterine cavity as affected by varieties of intrauterine contraceptive devices. Fertil. Steril., 37: 263-269.

Skangalis, M; Swenson, CE; Mahoney, CJ and O'Leary, WM (1979). The normal microbial flora of the baboon vagina. J. Med. Primatol., 8: 289-297.

Steel, C; Ratterree, M and Fidel, PL (1999). Differential susceptibility of two species of macaques to experimental vaginal candidiasis. J. Infect. Dis., 180: 802-810.

Tregier, A and Homburger, F (1961). Bacterial flora of the mouse uterus. Proc. Soc. Exp. Biol. Med., 108: 152-154.

Viana, D; Selva, L; Callanan, JJ; Guerrero, I; Ferrian, S and Corpa, JM (2011). Strains of *Staphylococcus aureus* and pathology associated with chronic suppurative mastitis in rabbits. Vet. J., 190: 403-407.

Watts, JR; Wright, PJ and Whithear, KC (1996). Uterine, cervical and vaginal microflora of the normal bitch throughout the reproductive cycle. J. Small Anim. Pract., 37: 54-60.

Westwood, FR (2008). The female rat reproductive cycle: a practical histological guide to staging. Toxic Pathol., 36: 375-384.

Ypsilantis, P; Tsiligianni, Th and Karagiannidis, A (1996). The use of cytological examination of vaginal smears for the determination of domestic rabbit's estrous cycle. Bull Hellenic Vet. Med. Soc., 47: 186-190.