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***In vitro* Study on the Susceptibility to *E. coli* Adhesion in Ewes during the Follicular and Luteal Phases of the Estrous Cycle**

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Abstract: The present study examined the susceptibility of uterine mucosa of ewes under the influence of estradiol and progesterone to *E. coli* adherence and colonization *in vitro*. Uterine explants were collected during the follicular and luteal phases of at peak levels of estrogen and progesterone respectively. Samples were inoculated with 10^8 cfu of *E. coli* culture. Serial sampling of the uterine explants of both phases were done at 45, 90, 180 and 360 min post-inoculation. The samples were processed for scanning electron microscopic examination. The results revealed that bacterial colonization on the uterine explants during follicular phase was significantly lower ($p < 0.05$) than during the luteal phase. Maximum colonization was observed at 360 min post-inoculation for both the follicular and luteal phases. Bacterial colonization on the luteal phase uterine explants was more severe than that of the follicular phase and it seems to increase with time.

Key words: *In vitro*, *E. coli*, follicular phase, luteal phase, ewes

INTRODUCTION

The reproductive tract, in particular the uterus, undergoes dynamic changes during the estrous cycle and ovarian steroids mainly oblige these events (Fazleabas and Zuzana, 2002). These changes in physiologic state place variable demands on the immune defenses of the reproductive tract. While the immune defense protects the reproductive tract against potential harmful pathogens, it must also be selectively neutral to allogenic spermatozoa and supportive for a fetal placental unit (Wira and Kauschic, 1996). This immune defense is regulated by the ovarian hormones (Entrican *et al.*, 2006).

The adhesion of bacteria to mucosal surfaces is an important step in the pathogenesis of most infections in humans and animals (Sugarmans and Epps, 1982; Yoshikaza and Baba, 1985b; Sobel and Kaye, 1986) because it is a prerequisite for colonization of the host (Van-den Bosch *et al.*, 1980). The mucosal surface possesses a diverse array of antimicrobial defense mechanisms including physical barrier (e.g., mucus) to bind and wash away microorganisms, nutrient-depriving molecules (e.g., lactoferrin, degradative enzymes, antimicrobial peptides, epithelial cell surface mucin and glycoproteins), which prevent adhesion of microorganisms to cell surface receptors for generation of the immune response (Pearson and Brownlee, 2005).

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In cyclic ewes, intrauterine inoculations of *Actinomyces pyogenes* and *Escherichia coli* led to the establishment of uterine infections at the point of the estrous cycle when progesterone concentration peaks. These infections did not occur at peak estradiol concentration (Ramadan *et al.*, 1997; Lewis, 2003; Seals *et al.*, 2003). In addition, many earlier studies proved that the immune systems of the ewes were lowered during luteal phase than during the follicular phase. In order to prove this, the same environment of the hormonal influences the uterus was created and challenged with the bacterial infection *in vitro*.

Steroid hormone action on bacteria infections may be mediated via the washing-out of microorganisms through cervical and vaginal discharges of inflammatory fluids (Lewis, 2003). However, the function of the hormones in modifying cell surface receptiveness to pathogenic bacteria attachment is yet to be elucidated (Yoshikazu and Baba, 1985b). Thus, the objective of this study was to determine the effect of estradiol and progesterone on the susceptibility of the uterine mucosa to *E. coli* colonization *in vitro*.

MATERIALS AND METHODS

Animals

The experiment was conducted from July to September 2008 (duration of 3 months) in Farm 2, Universiti Putra Malaysia. A total of 12 adult ewes (breed Malin, Malaysian Indigenous), age between 15-20 months old; mean weight \pm SEM of 17.3 \pm 0.42 kg, null parity with 1.04 ovulation rates (Azmi *et al.*, 1993) were used in this study. The ewes were kept in a raised slatted-floor house with open air and fed with chopped grasses of *Brachiaria decumbens* and water provided *ad libitum*. The ewes were supplemented with commercial pellet 0.5 kg/animal/day. The house ambient temperature was 26.8 to 32.8°C and humidity was 84.90 to 98.40%.

Estrus was synchronized using the progesterone sponge (Chronogest® 40, Intervet International, AUS.) containing 40 mg flugestone acetate. The sponge was inserted into the ewe's vagina, removed on day 14 and subsequently given an intramuscular injection of 350 - 450 IU of pregnant mare serum gonadotropin (PMSG) (Folligon®, Intervet International, AUS). Estrus was detected and confirmed by using bucks at 24 and 48 h following sponge withdrawal and with the presence of estrus signs.

Blood samples were collected on alternate days via jugular venipuncture. Hormonal profiles, estrogen (estradiol 17 β) and progesterone were determined using the RIA technique. Ewes at luteal and follicular phases were slaughtered when the blood progesterone and estradiol concentrations were at their peaks.

The study protocol was approved by the Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Inoculum Preparation

A pure, field strain *E. coli* isolated from the vaginal swabs were grown on blood agar at 37°C for 24 h. Thirty colonies were selected and seeded into 100 mL Brain-Heart Infusion (BHI) broth (OXOID) and incubated at 37°C for 18 h. The broth culture was standardized to contain 10⁸ cfu using McFarland turbidity standard.

Experimental Procedures

The ewes were slaughtered at the peak levels of blood progesterone and estradiol for the luteal and follicular phases, respectively. The uterus was removed and the uterine mucosa was obtained and maintained *in vitro* as explants in 12-wells tissue culture plates containing culture media. The protocol was according to the modified method of Effendy *et al.* (1998) and Al-Haddawi *et al.* (2000).

The collected uterine mucosae were diced to 1 mm³ and placed in a sterile container containing Hank's nutrient media and incubated in Gibco's BRL minimum essential media (MEM), (Sigma®) (containing 10% antibiotic-antimycotic, 10000 Units mL⁻¹ penicillin G sodium, 10000 µg streptomycin and 25 µg mL⁻¹ amphotericin B as fungizone®) at 37°C in 5% carbon dioxide.

After 24 h incubation, the uterine explants were removed, rinsed three times in MEM without antibiotics and immersed again in 3 mL MEM. The uterine explants of both follicular and luteal phases were divided into 2 groups each: (1) negative control group, without bacteria inoculation and (2) treated group, where the uterine explants were inoculated with 3 mL of inoculum containing 10⁸ cfu of live *E. coli* culture and incubated at 37°C in 5% carbon dioxide.

The *E. coli* inoculum used to challenge the explants was re-cultured onto blood agar to ensure that the bacteria were alive and the inoculum uncontaminated. Triplicate explants were collected from each plate at 45, 90, 180 and 360 min post-inoculation and processed for scanning electron microscopy.

Scanning Electron Microscopy

Uterine explants samples fixed in 4% glutaraldehyde for 4 h were washed in 0.1M sodium cacodylate buffer. Subsequently, the samples were post fixed in 1% osmium tetroxide, dehydrated in serial dilutions of ascending grades of acetone, critical-point-dried, sputter-coated with gold palladium and examined under SEM (JEOL 6400) at 15 kV.

Attached bacteria were counted on the electron micrographs captured from 15 fields per group and for each incubation period. The rate of bacterial colonization on the explants was recorded by the method described by Pijoan and Trigo (1990). This was based on the number of bacteria present on the explants and scored as follows: 0 (no colonization); 1 (mild colonization), single bacteria were found scattered throughout the uterine surface; 2 (mild to moderate colonization), moderate presence of bacteria in focal areas of the uterine tissues; 3 (moderate colonization), moderate numbers of bacteria were found throughout the uterine surface; 4 (moderate to severe colonization), abundant bacteria were found focally throughout the entire uterine surface; 5 (severe colonization), abundant bacteria were found on the entire uterine surface. The presence of *E. coli* on the explants was identified by their rod-shape and size, 1 µm in width, 2-4 µm in length.

Statistical Analysis

Data were analyzed using SPSS version 15.0. Bacterial scoring was analyzed using 2-way ANOVA to determine the effect of different time duration of post-inoculation between the follicular and luteal phases. All statistical analysis was conducted at 95% confidence level and differences of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The hormonal profile of the ewes showed that the follicular phase occurred between 24-36 h after sponge removal (Fig. 1). The average highest estradiol concentration was at day 2 and the progesterone concentration at day 11. The ewes were slaughtered on these respective days for sampling purposes.

The mean score of *E. coli* colonization on the uterine explants *in vitro* is presented in Fig. 2. *E. coli* colonization was significantly higher ($p < 0.05$) in the luteal phase than the follicular phase at all post-inoculation time intervals. However, the *E. coli* colonization was

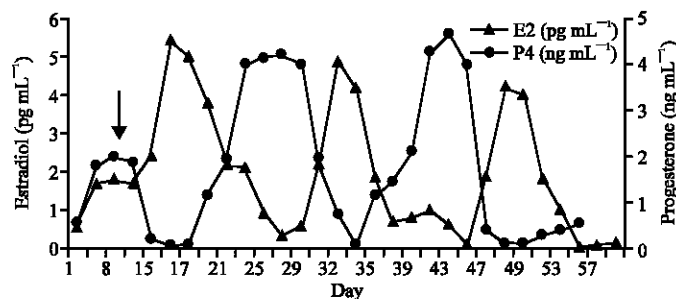


Fig. 1: The graph shows plasma estradiol and progesterone concentrations following estrous synchronization. The progesterone sponges (Chronogest® 40) were removed on day 14 (indicated by the arrow)

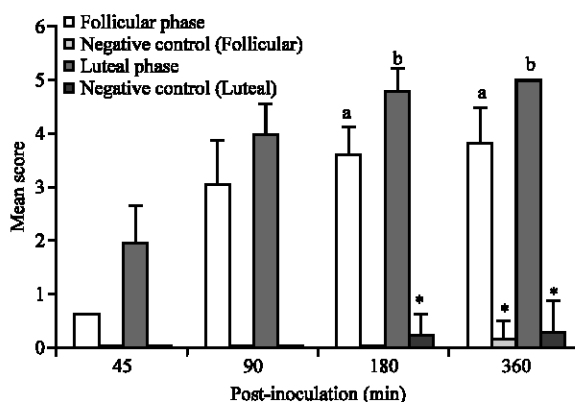


Fig. 2: *In vitro* *E. coli* colonization on the follicular and luteal phase uterus explants of ewes post-inoculation with the bacteria. ^{a,b}Means with different superscripts within postinoculation period differ significantly at $p = 0.05$

not significantly different ($p > 0.05$) between the negative control and inoculated specimens for both groups at all post-inoculation intervals. There were no bacteria found on the negative control specimens at 45 and 90 min post-inoculation for both the luteal and follicular groups. However, a few colonies of bacteria were found on the negative control luteal phase specimens at 180 and 360 min post-inoculation. Nevertheless, only the negative control follicular phase specimens showed bacteria colonies at 360 min post-inoculation. There was no significance difference ($p > 0.05$) in bacterial colonization between 180 and 360 min post-inoculation specimens. Maximum colonization of *E. coli* on the uterine explants from both groups was observed at 360 min post-inoculation.

Uterine explants of the follicular phase group showed either absence or very little colonization at 45 min post-inoculation (Fig. 3a). On the contrary, there were scattered individual bacteria with occasional moderate colonization of the explants of the luteal phase group at 45 min post-inoculation (Fig. 3b). Marked increased in the number of attached bacteria were observed at 90 min post-inoculation in both groups (Fig. 4a, b). Maximum colonization of *E. coli* was observed at 360 min post-inoculation for both the luteal and follicular phase explants, but this was not significantly different ($p > 0.05$) from 180 min post-inoculation (Fig. 5). At 180 and 360 min post-inoculation, there was rapid multiplication of

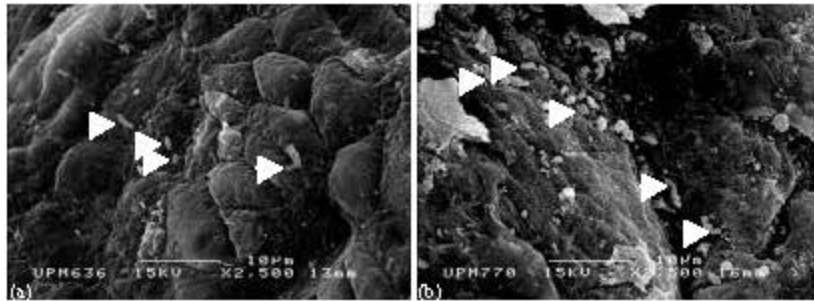


Fig. 3: Scanning electron microscopy show the uterine explants of ewes infected with *E. coli* at 45 min post-inoculation. (a) A few scattered individual bacteria (arrows) seen attached to the mucosa surface in follicular phase uterus. (b) Mild to moderate colonization of *E. coli* (arrows) onto the uterine mucosa of uterine explant in luteal phase uterus

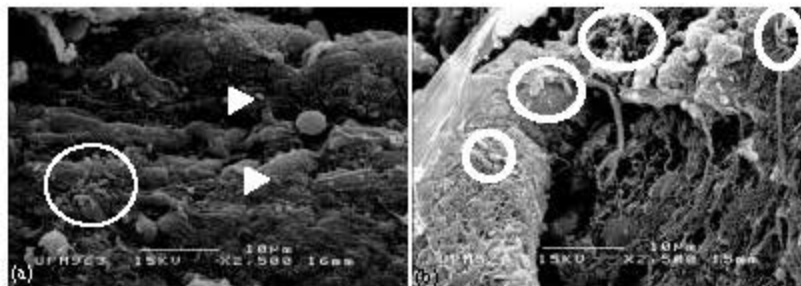


Fig. 4: Scanning electron microscopy show the uterine explants of ewes infected with *E. coli* at 90 min post-inoculation. (a) Moderate colonization of bacteria attached to the mucosa surface of uterine explant in follicular phase (circle and arrows). (b) Moderate to severe colonization of *E. coli* onto the uterine mucosa of uterine explant in luteal phase (circles)

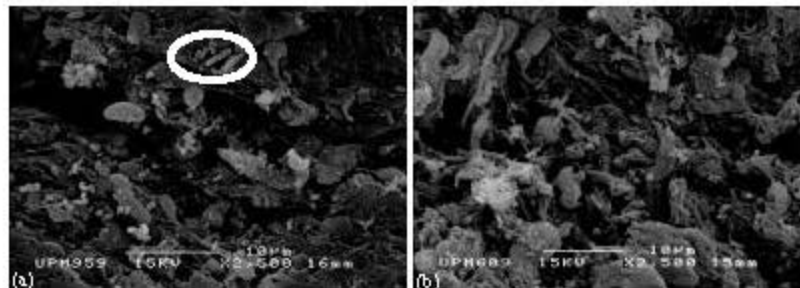


Fig. 5: Scanning electron microscopy show the uterine explants of ewes infected with *E. coli* at 180 min post-inoculation. (a) Moderate to severe colonization of bacteria attached to the mucosa surface of uterine explants in follicular phase uterus. Note some of the bacteria may undergo multiplication (circle). (b) Severe colonization of *E. coli* onto the uterine mucosa and note more massive tissues destruction of uterine explants in luteal phase uterus

bacteria for both groups. Furthermore, the luteal phase uterine explants at 360 min post-inoculation showed severe colonization and massive mucosal tissues destruction (Fig. 5b).

An overwhelming number of infectious diseases are initiated by bacterial colonization of the mucosal surfaces of the urogenital, gastrointestinal or respiratory tracts. Mucosal colonization by bacteria is preceded by bacterial attachment to epithelial cells or to mucin coating the mucosal cells that act as releasable decoy ligands for microbes attempting to anchor themselves to the glycocalyx (Linden *et al.*, 2008).

In the uterus, the bactericidal activities not only vary with stage of the estrous cycle but also dependent on the activity of ovarian hormonal level (Hawk *et al.*, 1961; Yoshikazu *et al.*, 1984; Matsuda *et al.*, 1985; Ramadan *et al.*, 1997; Bondurant, 1999; Seals, 2003). The hormones influence bacterial opportunistic adherence and may be a major factor in the pathogenesis of some infectious diseases (Entrican *et al.*, 2006).

Results observed in this study are in agreement with the *in vivo* study in rabbits and rats (Yoshikazu and Baba, 1985a; Yoshikazu, 1985) where the course of uterine infection when these animals were inoculated with *E. coli* was influenced by the stage of the estrous cycle. In these studies, the number of *E. coli* was reduced more rapidly at the follicular phase than the luteal phase. In the rat bladder and vaginal epithelial cells *E. coli* adherence corresponded with the peak levels of estrogen in the serum and urine (Van-den Bosch *et al.*, 1980).

Preliminary observations by Yoshikazu and Baba (1985b) in rats suggested that estradiol may reduce the susceptibility of the endometrial epithelium to *E. coli* adhesions by altering the nature of endometrial epithelium, thus preventing purulent endometritis. The researchers found out that *E. coli* adhered well to endometrial cells of ovariectomized rats but not estradiol-treated rats. However, the mechanism of bacteria adhesion remains to be determined.

Various reports have suggested that antibodies particularly IgA, play an important role against infection by inhibiting bacterial adherence. The IgA and IgG have been found at higher concentrations in uterine luminal fluid and their levels are influenced by estradiol (Wira and Sandoe, 1977; Wira and Sandoe, 1980; Sullivan *et al.*, 1983, Yoshikazu and Baba, 1985b; Sobel and Kaye, 1986).

Progesterone inhibits the bactericidal activity of the uterus and estrogen that is concurrently secreted at the luteal phase promotes the inhibitory action of progesterone (Matsuda *et al.*, 1985; Richardson *et al.*, 1993). These studies also showed that progesterone treatment prolongs survival time of *E. coli* by significantly lowering the phagocytic activity heterophils. This could be the reason for the significantly longer survival time of *E. coli* inoculated in the ligated uterus of rabbits at the luteal phase than at the follicular phase (Matsuda *et al.*, 1985).

In present study, there was progressive increase in bacterial colonization of the uterus during the follicular and luteal phases with progression of time. Nevertheless, the rate of colonization is greater in the luteal phase than the follicular phase explants. Thus, the findings in the present study complement those of previous findings by Yoshikazu *et al.* (1984) and Yoshikazu and Baba (1985a, b). In addition, we would like to suggest that the higher rate of colonization of bacteria during luteal phase is facilitated by the more favorable environment on the surface of the mucosa, which could have a bearing on the survival of the fertilized ovum in the uterus. The changes observed in cell-receptiveness in the uterus are most probably due to modifications in the properties of the cell surface (Entrican, 2006).

Another factor that could probably influence bacterial colonization to the uterus is the increased secretory products by the secretory cells during luteal phase compared to the

follicular phase, which will trap more bacteria in the reproductive tract (Intan-Shameha *et al.*, 2008). On the contrary, instead of being trapped by the mucosal secretion, these bacteria accumulated and began to adhere and colonize the mucosal surface.

Adherence is an important virulent factor in infections. Bacteria of piliated strains adhere better to cells than the nonpiliated strains. There are two different mechanisms for bacteria adherence, that is mannose-sensitive adherence and mannose-resistant adherence by piliated strains. In certain circumstances, strains without pili and without hemagglutinating activity adhere well. However, in most cases adherence seems to be mediated by bacterial pili (Van-den Bosch *et al.*, 1980).

CONCLUSION

In the present study, bacterial colonization was found to progressively increased with time of inoculation in both the follicular and luteal phase explants. *In vitro* colonization of *E. coli* on uterine mucosal explants appeared to be influenced by hormonal status namely estradiol and progesterone. This study also showed that bacterial colonization on the uterine tissue explants of ewes at peak luteal phase was greater than at peak of follicular phase.

From the present observation, it could also be postulated that the hormonal changes within the physiological range influenced the adherence of the bacteria on the mucosal surfaces, which could be the key to the pathogenicity of opportunistic infectious diseases.

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