Influence of Uterine Inflammation on the Estrous Cycle in Rats

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Abstract. To investigate how uterine inflammation affects ovarian activity in rats, endometritis was induced and changes in the length of estrous cycle and serum concentrations of estradiol-17\(\beta\) (E\(_2\)) and progesterone (P\(_4\)) were examined. A suspension of \textit{Staphylococcus aureus} (bacterial solution) or iodine solution was infused into the uterine lumen at various estrous phases. When the bacterial solution was infused at estrus, metestrus, or the first day of diestrus, the following diestrus continued for 5 to 12 days. In the case of the iodine solution, regardless of the estrous phase of the infusion, the following diestrus continued for approximately 6 days. E\(_2\) concentration after infusion of each solution did not fluctuate largely and remained at a low concentration (around 5 pg/ml). P\(_4\) concentration was high (35–45 ng/ml) on the day following infusion, but decreased rapidly to baseline values within a few days and remained thereafter at a low level (around 5 ng/ml). It is assumed that the endometritis caused by biological or chemical stimulation raises the concentration of P\(_4\) to depress gonadotrophic hormone secretion, and hence this high P\(_4\) concentration might inhibit the growth of ovarian follicles.

Key words: Endometritis, Estradiol, Estrous cycle, Progesterone, Rat

Materials and Methods

Animals
Adult female Wistar rats, weighing more than 220 g, which were inbred in this laboratory, were used in all experiments. Vaginal smears were taken daily and only those animals showing 3 consecutive 5-day estrous cycles were used. They were kept in a temperature-controlled room (22 °C) under a standard lighting regimen (12 h light: 12 h darkness, lights off at 2000 h) and provided with food (standard lab rat chow) and water ad libitum. All experiments were carried out in accordance with ethical consent from Azabu University.

Experiment 1
Physiological saline, a suspension of *Staphylococcus aureus* (bacterial solution) or iodine solution was infused into the uterine lumen at proestrus (Phase I), estrus (Phase II), metestrus (Phase IV), the first day of diestrus (Phase V1), or the second day of diestrus (Phase V2), and the change of estrous cycle was observed by examination of the vaginal smear. The rats which received the physiological saline, bacterial solution, or iodine solution were categorized into Group PS, Group SA, or Group IS, respectively. The iodine solution contained 6 g iodine, 4 g potassium iodide and 70 ml methyl alcohol in 100 ml. The bacterial solution was prepared by dissolving *Staphylococcus aureus* colonies growing on sheep blood agar into physiological saline and arranging the concentration into 2–15 × 10^8 colony forming unit (CFU)/ml. The rats were anesthetized with a subcutaneous injection of 8 mg pentobarbital sodium (Nembutal, Abbott laboratory, Illinois, USA) at between 10 and 12 am. After midline laparotomy and exposure of the uterine horn, 0.03 ml of one of the inoculi was injected into both the uterine lumen via a No. 26 gauge needle. Then 2 mg crystalline benzyl penicillin potassium dissolved in 1 ml physiological saline was administered into abdominal cavity. After these treatments, the number of successive diestrus days was counted for 19 days.

Experiment 2
In Experiment 1, there was a tendency for the succeeding diestrus to be longest when the bacterial solution was infused into the uterine lumen at Phase IV. Hence, in Experiment 2, the bacterial solution or the iodine solution was infused into the uterine lumen at Phase IV in the same way as in Experiment 1. Following this, the concentration of estradiol-17β (E2) and progesterone (P4) in serum were measured and the uteri were observed for histological changes. The rats were sacrificed every day from the day following inoculation (the 1st day) for 8 days and blood samples and uterine tissues were collected. After anesthesia as described above and laparotomy at between 10 and 12 am, blood was collected with a No. 26 gauge needle from the abdominal aorta. The blood was allowed to clot at room temperature and centrifuged, after which the serum was removed and frozen at –18 °C until the hormone assay was performed. The uteri were fixed in 10% (v/v) formaldehyde, embedded in paraplasts, sectioned at 4 µm and stained with hematoxylin and eosin. Blood was also collected from intact rats at each estrous phase as a control. E2 and P4 were measured by radioimmunoassay (RIA), using kits purchased from Diagnostic Products Corp. (Los Angeles, CA, USA). These kits employ a solid-phase radioimmunoassay designed for the direct, quantitative measurement of estradiol or progesterone in serum and require neither extraction nor predilution. All procedures followed the manufacturer’s instructions. When measuring standard sera containing known amounts of steroids, recovery was 80–120% for both kits. The cross-reactivity of the anti-E2 antibody for E2, estrone, estriol, 17α-estradiol, androstenedione and progesterone were respectively 100, 10, 0.32, 0.017, <0.01 and <0.01%. The cross-reactivity of the anti-P4 antibody for progesterone, 5α-pregnan-3, 20-dione, 17α-hydroxyprogesterone, 5β-pregnan-3, 20-dione, 20α-dihydroprogesterone, testosterone, 5β-pregnan-3α-ol-20-one, androstenediol and 17β-estradiol were respectively 100, 9, 3.4, 3.2, 0.2, 0.1, 0.05, <0.05 and <0.05%. The intra-assay and inter-assay coefficients of variation of these assays were <7.0% and <8.1% for E2 and <8.8% and <9.7% for P4, respectively.

Statistical analysis
The mean numbers of successive diestrus days in each three groups after inoculation, and serum concentrations of E2 and P4 were analyzed using ANOVA, followed by Fisher’s protected least-significant difference post-hoc analysis. Thresholds
used to detect the significant difference were 5 or 1%.

**Results**

**Experiment 1**

The numbers of successive diestrus days in Group PS, Group SA, or Group IS are shown in Table 1. In Group PS, diestrus was extended by about only 1 day and continued for 3 days when physiological saline was infused at Phase I, Phase II, Phase IV, or Phase V1. However, the estrous cycle was not changed when physiological saline was infused at Phase V2. In Group SA, diestrus continued for 5 to 12 days when the bacterial solution was infused at Phase II, Phase VI, or Phase V1 and the extension of diestrus tended to be longest in the case of Phase IV, whereas the estrous cycle did not change when the bacterial solution was infused at Phase I or Phase V2. There were significant differences between Group PS and Group SA at Phase II, Phase IV, and Phase V1 (P<0.05). There were also significant differences between Phase I or Phase V2 and Phase II, Phase IV, or Phase V1 (P<0.01). In Group IS, diestrus lasted for about 6 days, whenever the iodine solution was infused. There was a significant difference between Group PS and Group IS at Phase I (P<0.01), but there was no significant difference between each estrous phase.

**Experiment 2**

$E_2$ and $P_4$ concentration in serum: Figure 1 shows $E_2$ concentration after inoculation of the bacterial solution or the iodine solution. In the intact controls, $E_2$ concentration was lowest at Phase II (below 10 pg/ml). At Phase IV, $E_2$ concentration began to increase and peaked at Phase I (46.6 ± 7.8 pg/ml). In Group SA, $E_2$ concentration did not fluctuate largely throughout the observation period and was maintained below the lowest level of the control rats (around 5 pg/ml). There were significant differences between the intact controls and Group SA on the 1st day (P<0.05), the 5th day (P<0.05), the 2nd day (P<0.01), and the 3rd day (P<0.01). In Group IS, $E_2$ concentration was maintained below the lowest level of the control rats as in group SA for only 5 days after infusion of the iodine solution, when there was a tendency for it to increase. There were significant differences between the intact controls and Group IS on the 1st day (P<0.05), the 2nd day (P<0.01), and the 3rd day (P<0.01). There was also a significant difference between Group SA and Group IS on the 8th day (P<0.05). There was no significant difference between each day in Group SA and in Group IS. Figure 2 shows $P_4$ concentration after inoculation of the bacterial solution or the iodine solution. $P_4$ concentration in intact controls fluctuated between 5 and 16 ng/ml and it was lowest at Phase V2 (4.8 ± 1.1 ng/ml) and peaked at Phase I (16.3 ± 4.6 ng/ml). In Group SA and Group IS, $P_4$ concentration was as high with a two to three fold increase in the peak levels compared to control rats, on the day following infusion (the 1st day), after which it decreased to the base line value of control rats on the 4th day in Group SA and on the 2nd day in Group IS and was never raised after that. There were significant differences between the 1st or the 3rd day and the 8th day in Group SA (P<0.05), and between the 1st day and the other days in Group IS.

Table 1. The numbers of successive diestrus days after inoculation of physiological saline, suspension of *Staphylococcus aureus*, or iodine solution into rat uteri at each estrous phase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>I</th>
<th>II</th>
<th>IV</th>
<th>V1</th>
<th>V2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group PS</td>
<td>2.7±(2,3,3)</td>
<td>3.3±(4,3,3)</td>
<td>3.3±(3,4,3)</td>
<td>3.0±(3,2,4)</td>
<td>2.0±(2,2,2)</td>
</tr>
<tr>
<td>Group SA</td>
<td>2.0±(2,3,1)</td>
<td>8.0±(10,6,8)</td>
<td>9.3±(7,12,9)</td>
<td>6.0±(5,7,6)</td>
<td>2.0±(1,2,3)</td>
</tr>
<tr>
<td>Group IS</td>
<td>6.0±(6,6,6)</td>
<td>6.7±(5,9,6)</td>
<td>5.0±(6,6,3)</td>
<td>4.7±(5,4,5)</td>
<td>5.7±(6,2,9)</td>
</tr>
</tbody>
</table>

The values are means and individual values (in parenthesis). I: proestrus, II: estrus, IV: metestrus, V1: the first day of diestrus, V2: the second day of diestrus. $ab$ The values with different superscripts in a column differ significantly (P<0.05). $cd$ The values with different superscripts in the same group differ significantly (P<0.01).
There was no significant difference between the two groups on the same day.

Histological changes in uteri: In Group SA, moderate cellular infiltration, mainly consisting of neutrophils, was observed in the lamina propria mucosae on the 1st day, and the structure of the lamina propria mucosae collapsed on the 2nd day. On the 4th day, fibrocyte infiltration began (Fig. 3, A). Vacuolation, cornification and stratification were observed in luminal and glandular epithelial cells. Although these changes were moderate on the 1st day, they became gradually clear from the

(P<0.05).
3rd day and continued during the observation period. In group IS, severe infiltration of neutrophils into the lamina propria mucosae and uterine lumen were observed on the 1st day (Fig. 3, B). Uterine epithelial cells were separated from lamina propria mucosae and uterine glands contained inflammatory cells, however the uterine epithelium recovered normally and neutrophils in the uterine lumen had disappeared by the 3rd day. Infiltration of inflammatory cells into the lamina propria mucosae and uterine glands continued throughout the observation period, however they started to reduce from the 5th day. The vacuolation, cornification, and stratification in uterine epithelial cells and fibrocyte infiltration into the lamina propria mucosae, as was observed in group SA, did not occur in group IS.

Discussion

Infusion of the iodine solution into uteri at phase IV caused severe infiltration of inflammatory cells into the lamina propria mucosae, even on the 1st day. However, the change to the endometrium after infusion of the bacterial solution was moderate on the 1st day and then worsened gradually from the 3rd day becoming severe. These findings indicate that an iodine solution can induce inflammation on the endometrium immediately after infusion by chemical stimulation. However, in the case of the bacterial solution it seems that bacteria introduced into the uterus needs to settle there and proliferate to cause metritis. The defense mechanism of the uteri against bacterial infection is influenced with ovarian hormones and estrogen may act positively in relation to this [5, 25]. Hence, bacterial infection may not have been established in the cases when the bacterial solution was infused at phase I or phase V2, when E2 concentration is high. This may explain the phenomenon that the iodine solution could prolong diestrus whenever it was infused, but the bacterial solution could not when it was infused at phase I or phase V2. The histological changes of the endometrium induced by the bacterial solution lasted longer than that caused by the iodine solution. This may be due to evacuation of bacteria from the uteri taking a longer time. In any case, the presence of endometritis seems to prolong diestrus in rats.

The mechanism as to how endometritis has an influence on ovarian activity has not been clarified to date. E2 concentration in Group SA did not fluctuate largely throughout the observation period and was maintained at approximately 5 pg/ml. In Group IS, it also remained low for 5 days after infusion, following which, it began to rise. E2

Fig. 3. Uterine sections after inoculation (hematoxylin). A: A uterine section after infusion of a suspension of Staphylococcus aureus on the 4th day. Arrow indicates fibrocyte infiltration. B: A uterine section after infusion of the iodine solution on the 1st day. Severe neutrophilic infiltration of the uterine lumen and lamina propria mucosae were observed. Arrow indicates neutrophils in the uterine lumen. × 100.
concentration remains at baseline values of 7 pg/ml after ovulation until the next follicles develop [24]. Accordingly, it is speculated that folliculogenesis did not occur in Group SA or began after the 5th day in the Group IS. The existence of bacteria in the uterus after parturition can depress folliculogenesis and secretion of estradiol [17, 23]. It has been suggested that this depression of folliculogenesis by the uterine infection may be due to endotoxin produced in the uterus which could suppress the secretion of GnRH [1, 2, 18]. Battaglia et al. [1, 2] demonstrated that endotoxin acted on follicles directly and interfered with estrogen synthesis and the lack of estrogen suppressed the secretion of LH and FSH. However, infusion of the iodine solution which did not produce endotoxin also extended diestrus in this experiment. Hence, we suspect that a factor other than endotoxin extends diestrus.

Kalra et al. [11] and Kaneko et al. [12] reported that the administration of progesterone to rats at proestrus inhibited the secretion of estrogen, which triggers the ovulatory LH surge, and prevented ovulation. As P4 concentration was high, with a two to three fold increase in peak concentration compared to control rats on the day following infusion, we assume that the inoculation into the uterus stimulated progesterone secretion and suppressed ovulation.

The ovaries and adrenal cortex are considered to be the source of progesterone in blood. Uterine infection can prolong corpus luteum lifespan in cows, horses, and rabbits [3, 6]. However, rats do not have a luteal phase in the normal estrous cycle. Rivier et al. [20] and Rivest et al. [21] administered interleukin-1 (IL-1) into the brain ventricles of rats and found prolongation of diestrus. At this time, the volumes of corpora lutea and plasma progesterone levels were significantly increased. Furthermore, Rettori et al. [19] reported the release of prolactin, which stimulates corpora lutea, by IL-1 administration into the brain ventricles of rats. There is a possibility that the endometritis might induce release of prolactin via some cytokines. In rats, however, exposure of luteal cells to diurnal prolactin surges induces pseudopregnancy and diestrus follows for about two weeks. In this study, P4 concentration in Group SA and Group IS after uterine inoculation was high, but decreased rapidly and remained at low concentrations subsequently. This shows that the lifespan of the corpus luteum was not prolonged and that pseudopregnancy was not induced. Alternatively, stress stimulates progesterone secretion from the adrenal cortex in rats [7, 14] and this secretion depends on corticotrophin releasing hormone (CRH) from the hypothalamus [22]. CRH also acted on cells directly producing GnRH and suppressed secretion of GnRH [9, 13]. The high P4 concentration and low GnRH as a result of metritis might be responsible for ovarian quiescence. However, Nequin et al. [14] reported that E2 concentration also increased in addition to P4 concentration under a surgical stress condition, although this was not observed in this study.

In this study, the infusion of bacterial or iodine solution into the rat uterus increased P4 concentration and suppressed ovarian cyclic activity. In order to clarify the mechanism of this ovarian quiescence, the source of the increased P4 concentration remains to be elucidated. Pursuing this phenomenon in rats may give us information about the relationship between uteri and ovaries which transcends the difference of animal species.

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EF)FFS OF ENDOMETRITIS ON RAT ESTROUS CYCLE


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