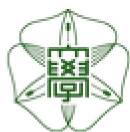

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—Technology Report—

Evaluating the electrical impedance and mucus-related gene expression of uterine endometrial tissues in mares

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Abstract. We investigated the electrical impedance of the reproductive tracts (vagina and uterine endometrial tissues) and the expression of mucus-related genes to identify the stage of the estrous cycle in mares. We first examined vaginal impedance in native Hokkaido mares during their estrous cycle and found no significant differences. However, impedance levels tended to decrease towards ovulation. Furthermore, we investigated the estrous cycle by measuring the electrical impedance of the uterine endometrial tissues obtained from carcasses of mares. We found that impedance levels in the endometrial tissues decreased in the regressed phase of the corpus luteum (CL). Expression of mucus-related genes (*ATP1A1*, *CFTR*, *AQP3*, and *AQP5*) varied at different stages of the estrous cycle. Among them, *AQP3* expression was consistent with previous reports. We concluded that electrical impedance in the uterine endometrial tissues of mares could be potentially used to verify the presence of active CL in horses for experimental purposes. However, further studies are needed to determine the reference value and to identify the day of the estrous cycle in mares.

Key words: Electrical impedance, Equine, Estrous cycle, Mucus-related gene

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Equine reproduction is seasonal, with mares undergoing estrous cycles from spring to early fall. It depends on the length of the day, and is quiescent in winter [1, 2]. In mares, ovarian follicular development and the subsequent corpus luteum (CL) formation and regression are associated with histological and histochemical changes in the reproductive tract, including the uterus. Conception leads to embryonic development in the uterus; whereas the endometrium releases prostaglandin $F_{2\alpha}$ to trigger CL regression if the mare does not conceive [3].

Several *in vivo* methods have been attempted to evaluate the estrous/menstrual cycle in female mammals. One such method uses electrical impedance to measure the electrical potential difference on the surfaces of the female reproductive tract, which include the cervix uteri, vagina, vaginal vestibule, and vulva [4]. Mucus secretion is considered to be one of the causes for impedance changes. Many domestic animals, including horses, exhibit changes in vaginal impedance during the estrous cycle [4]. However, we know very little about the exact nature of these changes in mares. Furthermore, these changes have not been measured in mare's uterine tissues. Although our previous research focused on examining the CL and uterus of mares across the estrous cycle [5, 6], mucus impedance and associated gene expression still remain unverified.

In this study, we tried to apply impedance measurements for experimental use. We first measured vaginal impedance in native Hokkaido mares to confirm the *in vivo* changes in impedance values through estrus. Next, we examined if the estrous cycle could be determined *in vitro* by measuring the electrical impedance coupled with mucus-related gene expression in the uterine endometrial tissues of mare's carcasses. These experiments were designed to form a better understanding of the reproductive physiology of mares.

To assess the feasibility of using electrical impedance to evaluate the estrous cycle in mares, we performed *in vivo* vaginal impedance measurements on native Hokkaido mares (n = 3) during the breeding season. Ultrasonography was performed daily to determine the day of ovulation (Day 0). In the present study, we found no significant difference in vaginal impedance levels in the native Hokkaido mares, possibly due to low sample size. However, impedance levels tended to decrease towards ovulation, and increased thereafter (Fig. 1). A decrease in vaginal impedance values during estrus has been reported in many domestic animals such as cows [7, 8], goats [9], and buffaloes

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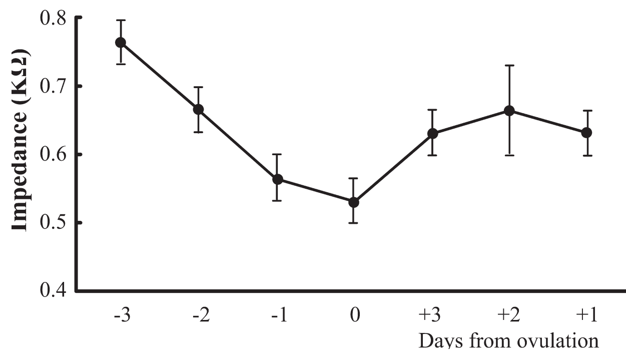

Fig. 1. Vaginal impedance levels in mares around the time of ovulation. Mean ± SEM values of vaginal impedance in native Hokkaido mares (n = 3) around the time of ovulation during the breading season (late April to late May in Hokkaido, ~N42°). The mares were monitored daily using ultrasonography to determine the day of ovulation (Day 0).

[10, 11]. The present study is the first to report vaginal impedance in mares during the estrous cycle since 1981 [12].

Next, we examined if measuring electrical impedance helped to determine the estrous cycle. The experiment employed uterine endometrial tissues from carcasses in mares. Using macroscopic observation as described previously [5, 6, 13], we classified the tissues as early luteal phase (corpora hemorrhagica (CH)), developing luteal phase (Dev-CL), middle luteal phase (Mid-CL), late luteal phase (Late-CL), and regressed luteal phase (corpora albicans (CA)) (Table 1). Next, we examined the electrical impedance during each phase (n ≥ 10 for each phase), and found that the impedance levels were lower in the CA phase when compared to all the other phases (Fig. 2; P < 0.05). Our observations demonstrated that the presence of active CL could be predicted by measuring the electrical impedance in uterine tissues, even after the uterine tissues were removed from the equine carcasses as samples for experimental use. Further research is required to establish reference values for each mare's breed so as to increase reliability.

Based on the impedance results, we hypothesized that the expression levels of the mucus-related genes would increase in the uterine tissues during the CA phase. Therefore, we quantified the relative mRNA levels of the following mucus-related genes: ATPase Na+/K+ transporting subunit alpha 1 (*ATP1A1*) [14], cystic fibrosis transmembrane conductance regulator (*CFTR*) [15], aquaporin (*AQP*) *3*, and *AQP5* [16]. Real-time PCR detected the mRNA of all the tested genes in the uterine endometrial tissues of the mares (Fig. 3).

The mRNA expression of *ATP1A1* in the CA phase tended to be lower when compared to both the CH and Late-CL phases; however, this difference was not significant (P < 0.1). To our knowledge, this is the first report to examine *ATP1A1* expression in mares. Further studies are necessary before we can fully understand the gene expression patterns and functions during estrus in mares.

Contrastingly, *CFTR* expression was high in the CH and Dev-CL phases when compared to the Mid-CL phase (P < 0.05). *CFTR* expression has been detected in the female reproductive tracts of rodents [17, 18] and humans [19], suggesting that CFTR may play physiological roles in female reproduction. In mice, maximum expression of *CFTR* mRNA in the uterine endometrium occurs three days after mating [18] to mediate bicarbonate secretion and the sperm fertilizing capacity [15]. Thus, our results supporting high *CFTR* expression during the CH and Dev-CL phases (i.e., post-ovulatory periods in mares) may reflect its contribution to mucus secretion for sperm interaction.

AQPs are involved in remodeling and hydrating the female reproductive tract [20, 21]. Here, *AQP3* expression increased dramatically from the Dev-CL to the CA phase (including estrus), whereas *AQP5* expression was significantly higher in the Dev-CL phase when compared to all the other phases (P < 0.05; Fig. 3). Although the sampling sites were different, our results were generally consistent with a previous study that reported peaked *AQP3* expression levels after the onset of estrus and decreased *AQP3* expression levels during the luteal phases in bovine cervix [21]. Increased *AQP3* expression levels at the onset of estrus may be related to water movement and mucus production in mares, similar to the role of the AQP proteins in maintaining cervical water balance during pregnancy and parturition in mice [20]. However, the function of the high *AQP5* expression levels in the Dev-CL phase remains unclear. More research is necessary to verify if the AQP5 expression patterns (high at Dev-CL and low at CA phase) are important for mucus production in mares.

Overall, our analyses showed that impedance levels in mare's uterine tissues decreased during the CA phase. Therefore, we conclude that electrical impedance in uterine tissues could be useful in determining the presence of active CL. However, in this study, we could not detect significant differences among the different estrous

Table 1. Morphological criteria to classify the ovarian estrous cycle in mares

Tissue	Criteria	Early (Corpora hemorrhagica) (CH)	Developing (Dev-CL)	Middle (Mid-CL)	Late (Late-CL)	Regressed (Corpora albicans) (CA)
Corpus luteum (CL)	Appearance	A large central cavity is filled with blood clot	CL starts to form, and blood clot remaining	CL is irregular, mushroom shaped or gourd shaped		CL is absent, or small and oblong structure
Follicle (FL)	Color	Dark red		Flesh colored	Buff colored	Straw colored
	Diameter of the largest FL (mm)		–15	15–20	30–35	> 35
Event			CL developing	CL maintained	CL regression	Estrus, Ovulation
Approximate day from after ovulation		Day 2–3	Day 4–6	Day 8–10	Day 12–16	Day 17–22

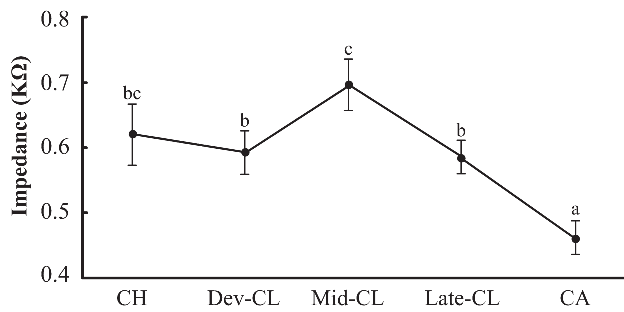

Fig. 2. Impedance changes in the uterine endometrial tissues of mare's carcasses during the estrous cycle. Mean ± SEM values of uterine endometrial tissue impedance in mares during the estrous cycle. The different letters indicate significant differences between each phase (P < 0.05) as determined by ANOVA, followed by Fisher's PLSD: early luteal phase (corpora hemorrhagica (CH)), developing luteal phase (Dev-CL), middle luteal phase (Mid-CL), late luteal phase (Late-CL), and regressed luteal phase (corpora albicans (CA)) (n = 10 from each luteal phase).

cycle stages in mares. This may be because of the limited samples. We recommend further studies examine the relationships between the mucus-related genes and mucus production to further evaluate the estrous cycle stages and/or to find characteristic changes in mares.

Methods

Animals

All animal procedures in this study were conducted in accordance with the Hokkaido University guidelines for the care and use of animals. Non-pregnant, mature native Hokkaido mares (n = 3, 5–16 years of age) were kept at the experimental farm of the Field Science Center for Northern Biosphere, Hokkaido University. Ultrasonography was performed daily to determine the day of ovulation. Vaginal mucus impedance was measured during the breeding season (late April to late May in Hokkaido, ~N42°) using an impedance checker (Muromachi, Tokyo, Japan; range, 0–19.9 kΩ, ± 1% ± 1 digit). Prior to measurement, the vulva was washed with soap, rinsed with water, and wiped with a paper towel. The impedance checker probe was then inserted into the vagina and pressed firmly against the vaginal wall. Impedance was recorded once the values stabilized.

Tissue collection

Non-pregnant uterine tissues from Anglo-Norman mares (n ≥ 10, 2–11 years of age, 600–1000 kg weight) were collected at the local abattoir in Kumamoto, Japan. Ovaries and uteri with macroscopic abnormalities, including ovulatory hemorrhagic follicles, were rejected. Uteri were classified via macroscopic observation of the CLs and follicles [5, 6, 13] into early luteal phase (corpora hemorrhagica (CH)), developing luteal phase (Dev-CL), middle luteal phase (Mid-

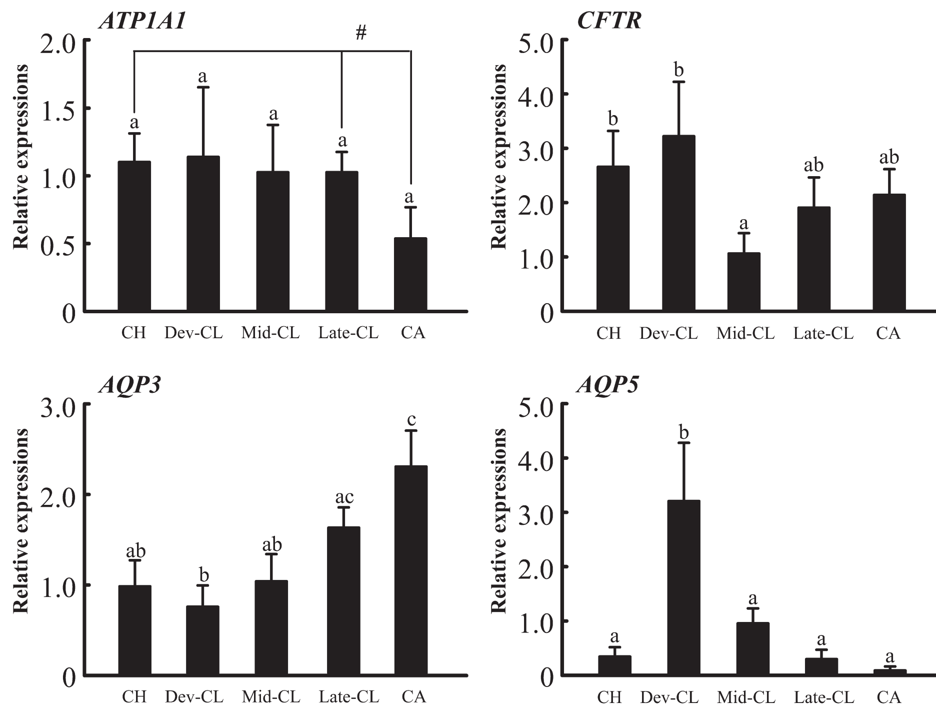

Fig. 3. Expression of mucus-related genes in the uterine endometrial tissues of mares during the estrous cycle. RNA extracted from the uterine endometrial tissues (n = 10 from each luteal phase) was subjected to real-time PCR analysis using the primers listed in Table 2. Real-time PCR analysis was used to estimate the expression levels of the mucus-related genes (*ATP1A1*, *CFTR*, *AQP3*, and *AQP5*) during the estrous cycle i.e., early (corpora hemorrhagica (CH)), developing (Dev-CL), middle (Mid-CL), late (Late-CL), and regressed CL (corpora albicans (CA)), in equine uteri. *GAPDH* mRNA was used as the internal control. All the data are represented as mean ± SEM. The different letters (a, b, c) indicate significant differences. The number sign (#) indicates a tendency, but not a significant effect (P < 0.1).

Table 2. Primers for real-time PCR

Name (GenBank accession No.)	Sequence (5'-3')	Annealing temperature (°C)	Product length (bp)
Mucus-related genes			
ATP1A1	F: GCGATTTCAGAGCATGGCAAC	54.0	146
(NM_001114532)	R: GTTAAGCCTCGGCTCAAGTCTG		
CFTR	F: CACGTTGAAAGCAGGTGGGA	55.0	124
(NM_001110510)	R: GGCCACAGCTCCAATCACAA		
AQP3	F: CGCCAACAACCAGCTTATAGTC	64.5	89
(AM182511)	R: CCATTGACCATGTCCAAGTGT		
AQP5	F: CCTGCTCTTCCCCAACTCG	67.0	65
(AJ514427)	R: GGCTCATACGTGCCCTTGAC		
Internal Control			
GAPDH	F: CGACCACTTTGTCAAGCTCA	56.0	135
(NM_001163856)	R: TCCTTCTCTTGCTGGGTGAT		

F: Forward, R: Reverse.

CL), late luteal phase (Late-CL), and regressed luteal phase (corpora albicans (CA)) (n ≥ 10 for each phase; Table 1). After the estrous cycle stage was determined, endometrial tissues ipsilateral to the CL or follicle were immediately frozen and stored at –80°C until they were processed for RNA extraction. During sample collection, the electrical mucus impedance of the uteri was also measured using an impedance checker. To measure the uteri mucus impedance, the impedance checker probe was inserted from the cervix to the uterus, and pressed against the uterine body. Impedance was recorded after stabilization. Note that electrical impedance was measured on the uterine body area of carcasses because the vaginal region was observed to be contaminated or damaged during sample collection.

RNA extraction and analysis

RNA extraction and analysis was performed as described previously [22]. Tissues were homogenized using a BioMasher® (Nippi, Tokyo, Japan) and total RNA was extracted using a NucleoSpin® RNA kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. For real-time PCR analysis, isolated RNA was reverse-transcribed to cDNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Osaka, Japan), and the resulting cDNA was stored at –30°C until use. The cDNA reaction mixture was diluted at a 1:3 ratio using deionized distilled water, from which 4 µl was used for each amplification reaction. The relative expression levels of the target genes were investigated via qRT-PCR using the LightCycler® 480 System II (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) containing a final concentration of 0.5 µM of each primer. Thermal cycling conditions were as follows: 1 cycle at 95°C for 30 sec and 45 cycles at 95°C for 10 sec, the annealing temperature for each primer pair for 15 sec, and 72°C for 30 sec. Relative mRNA abundance was calculated based on the expression of *GAPDH* as a reference gene. Details of the primers are shown in Table 2.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data was analyzed using a one-way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference (PLSD) tests using the StatView statistical analysis software (Abacus Concepts, Berkeley, CA, USA). All differences with $P < 0.05$ were considered significant. $P < 0.1$ were regarded to indicate a tendency.

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