J. Dairy Sci. 101:1–5
https://doi.org/10.3168/jds.2017-14251

Hot topic: Pregnancy-induced expression of interferon-stimulated genes in the cervical and vaginal mucosal membranes

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ABSTRACT

In ruminants, IFN-tau (IFNT) is a pregnancy recognition signal secreted by the embryonic trophectoderm before implantation, and it induces the expression of IFN-stimulated genes (ISG) in the uterine endometrium and blood leukocytes. The expression of ISG in blood leukocytes could indicate the presence of a viable conceptus before return of the next estrus; however, expression levels have high variation for confirming pregnancy. We hypothesized that the secreted IFNT in the uterus would affect ISG expression in cervical and vaginal tissues because they are directly adjacent to the uterus. To prove the hypothesis, we investigated the expression of 3 ISG (*ISG15*, *MX1*, and *MX2*) in cervical and vaginal mucosal membranes collected from pregnant (n = 12) and nonpregnant (n = 11) lactating Holstein cows at 17 to 18 d after artificial insemination. Mucosal membrane samples of the cervical canal near the external os (cervix) and deep vaginal wall surrounding the external os (vagina) were collected separately by simply scraping with a curette on d 17 or 18 of pregnancy (d 1 = ovulation), at which time IFNT secretion into the maternal uterus is maximal. After pregnancy diagnosis on d 30 and 60, separately collected samples confirmed as pregnant and nonpregnant were used for evaluation of the expression of IFN-stimulated protein 15 kDa (*ISG15*) and myxovirus-resistance protein 1 and 2 (*MX1*, *MX2*) with quantitative real-time PCR. The collected mucosal membrane samples from cervix contained mostly cell clots showing membrane structure and a low content of blood cells. The expression levels of all 3 genes were significantly increased in pregnant cows compared with nonpregnant cows in both cervical and vaginal samples. These results suggest that increased expression of ISG in the cervix and vagina is a pregnancy-associated phenomenon and is highly affected by IFNT secreted from the conceptus through the uterus.

Key words: pregnancy, interferon-stimulated genes, cervix, vagina

Hot Topic

Recently, the fertility of lactating cows has been decreasing, leading to substantial economic losses in the dairy industry. Moreover, the weakening of signs of estrus in the cow has made it difficult to detect estrus and perform AI with appropriate timing. Genetic selection for improving milk yield is considered a potential cause of infertility (Roelofs et al., 2010). Thus, it is important to detect nonpregnant cows before the return of estrus and perform AI without losing time. Interferon-tau (**IFNT**), produced by the ruminant trophectoderm, is a signal for the maternal recognition of pregnancy (Imakawa et al., 1987). It inhibits both the expression of oxytocin receptor and release of high-amplitude pulses of luteolytic $PGF_{2\alpha}$ in the uterine endometrium (Demmers et al., 2001) followed by the maintenance of corpus luteum. Production of IFNT is limited to the embryonic trophectoderm of ruminants during the peri-attachment period and it reaches its maximal level between d 17 and 19 of pregnancy (Ealy and Yang, 2009).

Induction of the expression of IFN-stimulated genes (**ISG**) is one of the most remarkable characteristics of IFNT. Interferon-stimulated genes such as IFN-stimulated protein 15 kDa (*ISG15*) and myxovirus-resistance protein 1 and 2 (*MX1*, *MX2*) are substantially induced in the uterine endometrium via the IFNT signaling pathway (Mansouri-Attia et al., 2009). Detection and measurement of these ISG could be a good indicator of

Received December 5, 2017.
Accepted May 22, 2018.
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early pregnancy determination that shows the existence of a conceptus in the uterus; however, direct collection of uterine tissues for ISG detection is impractical because it has a harmful effect on the conceptus and uterine condition. Several reports have shown high ISG expression in blood leukocytes of pregnant cows, which is expected to be an indicator of early pregnancy diagnosis (Green et al., 2010; Matsuyama et al., 2012; Yoshino et al., 2018). Although a significant difference in gene expression levels of ISG can be observed between nonpregnant and pregnant cows around d 18, detection and evaluation of blood leukocytes currently lacks reliability because of the high variation in the expression levels of mRNA of ISG between nonpregnant and pregnant cows even when IFNT secretion becomes maximum (Gifford et al., 2007).

Recent studies have shown that a pregnancy-specific increase of ISG is present not only in uterine tissues and blood cells, but also in other organs, including high expression levels of *ISG15* in the ovine corpus luteum at d 15 of pregnancy (Oliveira et al., 2008), *ISG15* and *MX1* expression in the bovine liver at d 20 of pregnancy (Meyerholz et al., 2016), and protein and mRNA of ISG15 in ovine bone marrow at d 16 (Yang et al., 2017). These studies suggest that organs other than the uterine tissue and blood leukocytes have pregnancy-specific responsiveness to type-I IFN that stimulates ISG expression through the bloodstream.

On the contrary, when focusing on the location of organs directly adjacent to the uterus, the cervix is physically open throughout the entire estrous and gestation period. This raises the possibility that factors secreted in the uterus move towards to the adjacent organs, causing some effects directly or indirectly. Therefore, we hypothesized that secreted IFNT in the uterus may affect extra-uterine tissues, such as cervical tissue, directly or indirectly through the reproductive tract. To prove the hypothesis, we investigated the expression of 3 ISG—*ISG15*, *MX1*, and *MX2*—in cervical and vaginal mucosal membranes collected from pregnant and nonpregnant lactating Holstein cows on d 17 to 18 after AI. We also measured the expression level of *ISG15* in peripheral blood leukocytes in pregnant and nonpregnant cows.

Lactating Holstein cows maintained at the Field Center for Northern Biosphere, Hokkaido University (Hokkaido, Japan) and Konsen Agricultural Experiment Station (Nakashibetsu, Hokkaido, Japan) were used for this experiment. The protocol was reviewed and approved by the Institutional Animal Care and Use Committee of National University Corporation Hokkaido University (approval no. 16-0019) and the Animal Care Committee for Laboratory Animals of the Konsen Agricultural Experiment Station (approval no. 20180312-3). Sampling was conducted on d 17 or 18 (d 0 = standing estrus and AI) of pregnancy by scraping the mucosal membrane with a curette (Figure 1B) from the cervical canal near the external os at approximately 3 to 5 cm deep (cervix; Figure 1A, white arrow) and in the deep vaginal wall surrounding the external os (vagina; Figure 1A, black arrow). Each sample was collected with a volume of approximately <100 μL (Figure 1B). Samples were collected from the vagina first, followed by the cervix, changing the curette in between. The overall time required for sample collection was less than 20 min on average. Blood samples were collected from the jugular vein at the same time into tubes containing EDTA-2Na (Terumo, Tokyo, Japan). Some mucosal membrane samples were immediately placed into

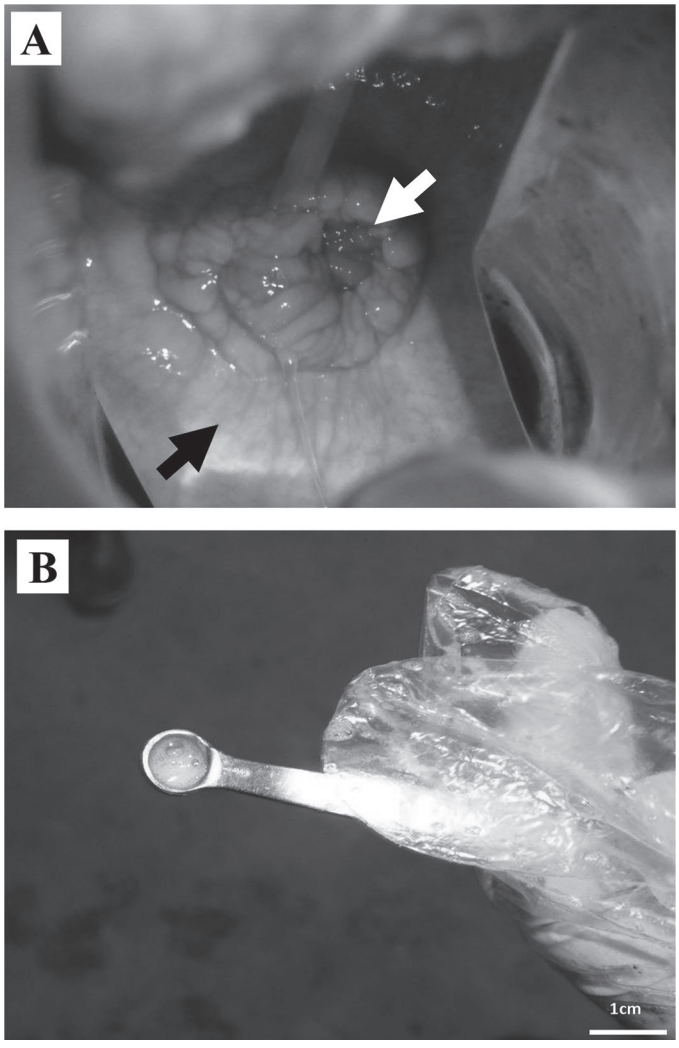

Figure 1. Sampling points of the extra-uterine sites of cows: (A) The white arrow shows the external os of the cervix (cervical samples); the black arrow shows the deep vaginal wall surrounding the external os (vaginal samples). (B) Sampling curette and collected mucosal membrane sample from cervix. Color version available online.